CELL CULTURE STUDIES

OF

OLFACTORY RECEPTOR NEURONS

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ABSTRACT

This study was conducted to investigate the extrinsic factors influencing neuronal differentiation in vitro. A cell culture model of olfactory receptor neurons (ORNs.) was used. In this study, ORNs were cultured on cortical astrocytes, olfactory bulb (OB) astrocytes, glial cells from the olfactory nerve fiber layer (ONL), and fibroblasts. Observations on the morphological differentiation of ORNs on these cellular substrata were made by scanning and transmission electron microscopy, and the extent of neurite outgrowth from these cells was quantified. The role of soluble factors in mediating olfactory neurite outgrowth on the cellular substrata was also investigated.

The results showed that ORNs differentiated into bipolar cells on cortical astrocytes, and their processes resembled axons and dendrites. Quantitative analysis indicated that on cortical astrocytes, OB astrocytes and ONL glial cells, the percentage of ORNs bearing neurites, neurite length, and the mean number of ORNs attached to substratum per coverslip were not significantly different among these three groups. Furthermore, the percentage of ORNs that extended neurites and the length of the neurites in these groups were significantly greater when compared to those on fibroblasts.

Immunofluorescent staining for cell adhesion molecules showed that cortical astrocytes expressed both N-CAM and N-cadherin, whereas on fibroblasts only N-cadherin was present. The absence of N-CAM on fibroblasts may account in part for the decreased percentage of cells with neurites.

Investigation of the effect of media conditioned by cortical

astrocytes (CMA) and fibroblasts (CMF) showed that olfactory neurite extension on cortical astrocytes was significantly reduced in CMF, suggesting that fibroblasts produce certain substance(s) which is inhibitory to olfactory neurite extension. The inhibitory substance(s) acts by adsorbing to the substratum.

Analysis of CMF showed that the inhibitory substance(s) had a molecular weight greater than 3,000 daltons. Heat treatment did not eliminate the inhibitory effect of CMF on olfactory neurite growth, suggesting that the substance(s) is either not a protein(s), or is a heat stable protein.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ara-C	Cytosine arabinoside
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CAM(s)	Cell adhesion molecule(s)
CHAT	Choline acetyltranferase
CM	Conditioned medium
CMA	Conditioned medium of cortical astrocytes
CMF	Conditioned medium of fibroblasts
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
DNAs	Deoxyribonuclease
ECM	Extracellular matrix
FITC	Fluorescein isothiocyanate
FGF	Fibroblast growth factor
FN	Fibronectin
GFAP	Glial fibrillary acidic protein
GLM	Glomerular layer
HBSS	Hank's balanced salt solution
H & E	Hematoxylin and eosin
HMF	HEPES-buffered Ca^{2+}- and Mg^{2+}-free saline supplemented with 1 mM $CaCl_2$
LN	Laminin
MEM-H	Minimum essential medium Eagle with 25 mM HEPES
MW	Molecular weight

N-CAM	Neural cell adhesion molecule
NGF	Nerve growth factor
OB	Olfactory bulb
OD	Optical density
OE	Olfactory epithelium
OM	Olfactory mucosa
OMP	Olfactory marker protein
ONL	Olfactory nerve fiber layer
ONSC	Olfactory nerve Schwann cells
ORN(s)	Olfactory receptor neuron(s)
PBS	Phosphate buffered saline
PLL	Poly-L-lysine
PNS	Peripheral nervous system
RT	Room temperature (20°C)
RT97	Anti-neurofilament antibody
SEM	Scanning electron microscope
TBS	Tris-buffered saline
TBS-Ca	TBS containing 1 mM $CaCl_2$
TEM	Transmission electron microscope

INTRODUCTION

ANATOMY OF OLFACTORY MUCOSA

In rodent species, olfactory mucosa (OM) covers the superior region of the nasal cavity, the posterior region of the nasal septum and the turbinates. It is composed of the olfactory epithelium (OE) and the underlying lamina propria. The neural components of OM consist of the olfactory receptor neurons (ORNs) and their axons. The cell bodies of ORNs are within the OE, while their axons extend into the lamina propria. The olfactory axons form bundles which penetrate the foramina of the cribriform plate to synapse with second order neurons in the olfactory bulb (OB) (Graziadei and Monti-Graziadei, 1979).

Olfactory epithelium is a pseudostratified columnar epithelium. Earlier descriptions indicate that the OE consists of ORNs, basal cells, and supporting cells (e.g. Rafols and Getchell, 1983; Costanzo and Morrison, 1989a). Recently a fourth type of cell, the microvillar cell, has been identified in the OE of man (Moran et al., 1982; Costanzo and Morrison, 1990) and rat (Rowley et al., 1989). The ratio of microvillar cells to ORNs is approximately 1 to 20 in the OE of rat (Rowley et al., 1989). In OE, the nuclei of supporting cells are located in the upper one third of the epithelium, while those of the ORNs are in the middle. The nuclei of basal cells lie adjacent to the basal lamina, while those of microvillar cells are close to the epithelial surface (Graziadei and Monti-Graziadei, 1978; Costanzo and Morrison, 1990)

The cell bodies of ORNs are pear-shaped and bipolar. A single, unbranched dendrite grows from the apical pole of each cell body,

ending in a swelling from which cilia of different lengths extend. The axons growing from ORNs form small intraepithelial fascicles which coalesce to become larger ones in the lamina propria (Costanzo and Morrison, 1989a, 1990). In most vertebrate species, the diameter of the ORN cell bodies is 5-8 μm, while the width of dendrites and axons are 1-2 μm and less than 0.5 μm respectively (Gasser, 1956; De Lorenzo, 1957; Graziadei, 1971).

Supporting cells are tall columnar cells. Microvilli extend from the mucosal surface of each cell body. The cell body forms a basilar expansion at the interface of the epithelium and the lamina propria (Rafols and Getchell, 1983). There are processes extending laterally from the cell bodies to surround or contact adjacent ORNs and supporting cells (Rafols and Getchell, 1983; Costanzo and Morrison, 1989a, 1990). Tight junctions form between supporting cells and adjacent ORNs just below the dendritic knobs (Kerjaschki and Hörandner, 1976). Supporting cells give physical support to the ORNs, and help to maintain the mucous layer on the epithelial surface (Rafols and Getchell, 1983; Getchell et al., 1984). They resemble cerebellar astrocytes morphologically and are postulated to serve as a guide for ORN development (Rafols and Getchell, 1983; Costanzo and Morrison, 1989a).

Microvillar cells have flask-shaped cell bodies. From the tapered apical aspect of each microvillar cell body, a cluster of microvilli grows into the nasal cavity, while from its base a fine process extends into the lamina propria. Microvillar cells may be secondary chemoreceptors as their axons have been demonstrated to travel with the

ORN axons to the OB (Rowley et al., 1989).

The lamina propria underlying the OE consists of loose connective tissue, numerous blood vessels and serous glands (Bowman's glands), and olfactory axonal bundles which are ensheathed by olfactory nerve Schwann cells (ONSC) (Rafols and Getchell, 1983; Costanzo and Morrison, 1989a). Bowman's glands produce watery secretions onto the surface of the epithelium to dissolve odiferous substances. The ONSC possess some characteristics of astrocytes as well as peripheral Schwann cells. They contain the central type of glial fibrillary acidic protein (GFAP) (Barber and Dahl, 1987). Like peripheral Schwann cells which envelop unmyelinated axons, they are peripheral in location, reside in connective tissue, and extend processes to wrap around bundles of olfactory axons (Rafols and Getchell, 1983).

NEUROGENESIS AND DIFFERENTIATION OF OLFACTORY RECEPTOR NEURONS

The most prominent feature of the ORNs is that they are generated continuously throughout adult life (Graziadei and Monti-Graziadei, 1978, 1979).

The OE originates from the craniolateral portions of the olfactory placodes, which are a pair of ectodermal thickenings on the anterolateral surface of the embryonic head. In mouse, by embryonic day 10 (E10; E1 refers to the day when the dam is sperm-positive), the axons begin to grow from the cell bodies. The axons at this time have a diameter of 0.5-2.0 μm. At E11, axonal bundles are first seen, and later finer axons appear as they become sequestered into bundles (Cuschieri and Bannister, 1975). The axons reach the cerebral vesicles which evaginate to form the OB at E12, but synapses are first detected

only at E15 (Hinds, 1972; Hinds and Hinds, 1976). With increasing fetal age, the proportion of fine axons increase, and by E18, 95% axons are less than 0.5 μm in diameter (Cuschieri and Bannister, 1975). Dendrites which contain microtubules and clusters of centrioles begin to form at E11. At E12, terminal swellings of dendrites are seen while cilia are infrequent (Cuschieri and Bannister, 1975). At E17, cilia proliferate greatly from the dendritic knobs, which by this time possess no mitochondria, few free centrioles, and a few microtubules. In the perikarya of ORNs, abundant smooth and rough endoplasmic reticulums and lysosomes as well as enlarged Golgi complexes are present. Olfactory receptor neurons which resemble mature ones are present by E18 (Cuschieri and Bannister, 1975). In rat the developmental events generally proceed 2-3 days slower than those in mouse (Brunjes and Frazier, 1986).

In adult mouse and frog, autoradiographic and morphological studies on OE show that ORNs have a life span of approximately 30 days, and the basal cells can differentiate into mature ORNs in 8 days (Graziadei and Monti-Graziadei, 1978). Basal cells lie alone or in clusters in the basal region of the epithelium, producing neuroblasts which differentiate into ORNs. In regions where the genesis of ORNs is active there are more basal cells and young ORNs than mature ORNs, whereas in zones where neurogenesis is not active, mature ORNs predominate (Graziadei and Monti-Graziadei, 1978, 1979).

ANATOMY AND DEVELOPMENT OF THE NERVE FIBER LAYER OF THE OLFACTORY BULB

The adult OB can be divided into 6 layers from without inwards:

olfactory nerve fiber layer (ONL), glomerular layer (GLM), external plexiform layer, mitral cell layer, internal plexiform layer, and granule cell layer. Axons from the OE enter and form the ONL, and synapse with dendrites of the second order neurons, the mitral cells, at the GLM. The glial cells are the only cell type present in the ONL (Doucette, 1984).

The ONL glial cells are of two types which are different in morphology, function, and origin (Doucette, 1984, 1989). One type has typical structural features of astrocytes, and does not ensheath the olfactory axons (Doucette, 1984). The other type is responsible for enveloping the axons and is referred to as the ensheathing cells or peripheral glial progenitor cells (Doucette, 1984). The ensheathing cells are similar to Schwann cells as they extend processes to envelop bundles of axons, are spindle-shaped in vitro (Barber and Lindsay, 1982), and express the surface antigen for 217c which is a marker for Schwann cells (Fields and Dammerman, 1985). However, they also possess some characteristics of astrocytes. They contain the central type of GFAP (Barber and Dahl, 1987) and form part of the glial limitans on the surface of the OB (Doucette, 1989). They are not oligodendrocytes or microglia, because neither of these cells have GFAP nor do they form glial limitans of the brain (Mori and Leblond, 1969, 1970).

Morphological studies on ONL development suggest that the two cell types form at different developmental stages. In mouse the rostroventral portions of the paired cerebral vesicles evaginate to form the OB primordium at about E13-14. Over the external surface of the bulb primordium a glial limiting membrane is formed by the

cytoplasmic processes of astrocyte precursors in the bulb (Doucette, 1989). When the olfactory axons from OE reach the bulb primordium, cells enveloping the axons (presumptive ensheathing cells) grow over the glial limitans to form the ONL (Doucette, 1989). Later the axons enter the presumptive GLM, and the glial limitans between ONL and the presumptive GLM disappears. Most of the presumptive ensheathing cells remain in the ONL although some of them may enter the GLM with the axons (Marin-Padilla and Amieva, 1989). The presumptive ensheathing cells differentiate into the ensheathing cells in the ONL (Doucette, 1989). The ensheathing cells also form the new glial limitans over the ONL with the astrocytes in this layer (Doucette, 1989).

The astrocytes in ONL, however, are of central origin. The ONL glia of newborn mouse consists of the ensheathing cells only (Doucette, 1990). The astrocytes are thought to migrate into the ONL from deeper layers of the bulb after birth, occupying the spaces among the ensheathed axonal bundles (Doucette, 1990). This proposal is consistent with the report that the ONL of rat increases in size up till the postnatal day 30 (P30; P0 refers to the first 24 hours after birth) to the end of the second year (Hinds and McNelly, 1977).

It has been demonstrated that the ingrowing axons of the ORNs can promote the proliferation of cells in the cerebral vesicles during OB formation (Piatt, 1951; Stout and Graziadei, 1980). Olfactory axons can also organize the GLM (Stout and Graziadei, 1980).

FACTORS INFLUENCING NEURONAL DIFFERENTIATION

It has been proposed that neurogenesis and differentiation of ORNs are controlled by both genetic and environmental factors (Farbman,

1990). The continuous renewal of the ORNs throughout adult life appears to be determined by intrinsic factors (Graziadei and Monti-Graziadei, 1978), while recent data show that changes in the environment of the ORNs can modulate the life span and the rate of genesis of the neurons (Hinds et al., 1984; Farbman et al., 1988).

Studies concerning neuronal differentiation have been conducted at molecular level. The major environmental factors affecting neuronal differentiation which have been identified include: extracellular matrix (ECM) molecules, cell adhesion molecules (CAMs), and soluble diffusible molecules. The first two kinds of molecules are associated with cell surface.

(1) EXTRACELLULAR MATRIX MOLECULES

Extracellular matrix molecules refer to the constituents of the material occupying the spaces between cells. Examples of ECM molecules are laminin (LN), fibronectin (FN) and cytotactin (also known as tenascin and contactin), all of which are glycoproteins (Timpl and Dziadek, 1986).

Laminin has a molecular weight (MW) of about 850 kD, and is a major component of basement membrane (Engel et al., 1981). Immunohistochemical studies have shown that LN is present in the developing brain (Liesi, 1985b; Letourneau et al., 1989) and in the optic nerve (McLoon et al., 1988). In dissociated cell culture, LN is an effective promotor for the neurite outgrowth of some PNS neurons such as embryonic chick dorsal root and sympathetic ganglia neurons, and CNS neurons such as retina and spinal cord neurons (Rogers et al., 1983). It has also been shown that cultured astrocytes can secrete LN

(Liesi et al., 1983). Laminin is expressed on some GFAP-positive cells in the ONL of adult rat. The processes of these cells are in apposition to olfactory axons, suggesting that the LN is produced by the ensheathing cells (Liesi, 1985a).

Laminin mediates cell-matrix adhesion and interaction through binding to cell receptors (Timpl, 1989). The cell receptors for LN are integrins which are a family of integral membrane proteins. The integrins bind to cytoskeleton indirectly, and cytoskeleton-associated proteins such as talin and vinculin are required as linkers (Burridge et al., 1988).

Fibronectin may affect the formation of the cerebral cortex as suggested by its distribution pattern in embryonic brain (Pearlman et al., 1986; Stewart and Pearlman, 1987; Chun and Shatz, 1988). It can also promote the neurite outgrowth of embryonic chick dorsal root and sympathetic ganglia neurons, whereas retina and spinal cord neurons cannot extend neurites on it (Rogers et al., 1983).

Cytotactin has a MW of about 200 kD, and is widely distributed in both neural and non-neural tissues. It is produced by glia and mediates glia-neuron interactions (Grumet et al., 1985). There are conflicting reports concerning the effect of cytotactin on neuronal growth and migration (Chuong et al., 1987; Bronner-Fraser, 1988; Grierson et al., 1990).

(2) CELL ADHESION MOLECULES

In the nervous system the major CAMs include the neural cell adhesion molecule (N-CAM), L1 and N-cadherin, all of which are integral membrane glycoproteins (Cunningham et al., 1983; Faissner et al., 1985;

Hatta et al., 1988). The neural cell adhesion molecule has three major isoforms with MW of 120, 140 and 180 kD respectively (Rougon et al., 1982; Chuong and Edelman, 1984). Immunohistochemical studies show that the MW 180 kD isoform of N-CAM is colocalized with spectrin, a membrane-cytoskeleton linker protein, suggesting that the adhesion may transfer signals from the cell surface to the cytoskeleton (Pollerberg et al., 1987). It mediates some morphogenetic processes such as lamination of retina during embryonic development (Silver and Rutishauser, 1984; Thanos et al., 1984). It persists on most CNS and PNS neurons in the adult (Langley et al., 1983; Nieke and Schachner, 1985). It is also present on cultured astrocytes (Keilhauer et al.,1985).

L1 is of MW 200 kD. It is present on postmitotic neurons (Rathjen and Schachner, 1984). It is also involved in morphogenetic processes such as the migration of cerebellar granule cells (Lindner et al., 1983).

N-cadherin (also known as A-CAM) colocalizes with the actin cytoskeleton (Hirano et al., 1987), and is associated with the intercellular adherens-type junctions such as zonula adherens which involve actin filaments (Volk and Geiger, 1986a, b). N-cadherin has a MW of 130 kD, and specifity is determined by the amino terminal 113 amino acid region of the extracellular domain (Rutishauser, 1989; Nose et al., 1990). N-cadherin seems to be present on all neurons in the brain of chick embryos (Hatta et al., 1985; Hatta and Takeichi, 1986). It may partipate in morphogenetic events as suggested by its expression in neural tube of chick embryo (Hatta and Takeichi, 1986).

Cell adhesion molecules mediate adhesion via a homophilic and/or a heterophilic binding. The former involves surface molecules of the same type, such as N-CAM binding to N-CAM, whereas the latter involves surface molecules of different types, such as LN binding to integrin. The neural cell adhesion molecule mediates homophilic, Ca^{2+}-independent adhesions between cells (Rutishauser et al., 1982; Sadoul et al., 1983; Keilhauer et al., 1985). The adhesion mediated by N-cadherin is also homophilic but Ca^{2+}-dependent (Hatta et al., 1985; Hirano et al., 1987). However, L1 mediates a homophilic, Ca^{2+}-independent neuron-neuron binding (Grumet et al., 1984; Keilhauer et al., 1985; Lemmon et al., 1989), and possibly a heterophilic neuron-glia binding (Grumet et al., 1984; Grumet and Edelman, 1988).

The neural cell adhesion molecule and L1 have been detected in the olfactory system of both embryonic and adult mice by immunofluorescence and immunoelectron microscopy (Miragall et al., 1988, 1989). In olfactory placode, N-CAM but no L1 is expressed on placodal cells. In the developing OM, both N-CAM and L1 are present on ORN cell bodies and axons, and on ONSC. In early postnatal stage and in adult, N-CAM is also present on dendrites, whereas L1 disppears from the cell bodies. The neural cell adhesion molecule is present on the axons and the glial cells in both developing and adult ONL, while L1 is expressed only on the axons.

The difference between ECM molecules and CAMs is not always distinct. For example, cytotactin, N-CAM and L1 belong to the immunoglobulin gene superfamily and have remarkably similar structures

and amino acid sequences (Cunningham et al., 1987; Moos et al., 1988; Ranscht, 1988). Cell adhesion molecules such as N-CAM and L1 may have overlapping localizations with ECM molecules (Martini and Schachner, 1986, 1988; Rieger et al., 1988). Both ECM molecules and CAMs interact with the cytoskeleton (Horwitz et al., 1986; Pollerberg et al., 1987; Hirano et al., 1987). It has been postulated that the mechanisms underlying the cellular responses to these two groups of molecules may not be fundamentally different (Lander, 1989).

(3) SOLUBLE DIFFUSIBLE MOLECULES

Soluble diffusible molecules generally refer to neurotrophic factors secreted by cells into the culture medium. These factors usually regulate some aspects of neuronal differentiation. The best understood neurotrophic factor is nerve growth factor (NGF), while in recent years others have been discovered: brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and fibroblast growth factor (FGF). Nerve growth factor and BDNF are target-derived, while CNTF and FGF are not (Barde, 1988; Thoenen, 1991).

Nerve growth factor is a polypeptide (Bueker, 1948; Levi-Montalcini and Hamburger, 1951; Cohen, 1960). It is generally agreed that NGF plays a critical role in the development of sympathetic and some sensory neurons in vertebrates (Levi-Montalcini and Angeletti, 1968; Levi-Montalcini, 1987). It has recently been demonstrated that NGF administration can increase the activity of choline acetyltransferase (CHAT) in neonatal rat forebrain and striatum, as well as in adult rat brain after injury (Gnahn et al., 1983; Hefti et al., 1984; Mobley et al., 1985; Williams et al., 1989). It has been

shown by immunoelectron microscopy that NGF protein is present within the ORNs of mouse embryo (Williams and Rush, 1988).

Brain-derived neurotrophic factor is a basic protein of MW 12 kD purified from the brain (Barde et al., 1982). It resembles NGF in structure (Leibrock et al., 1989), and supports the survival of sensory neurons in vitro (Lindsay and Rohrer, 1985; Davies and Lindsay, 1985). It can also support the survival of some neurons which are not responsive to NGF, such as retina neurons (Johnson et al., 1986).

Ciliary neurotrophic factor is an acidic protein of MW 20 kD (Adler et al., 1979; Barbin et al., 1984). It can support the survival of ciliary ganglion, sympathetic, sensory neurons and spinal motoneurons in vitro (Adler et al., 1979; Barbin et al., 1984; Manthorpe et al., 1986; Arakawa et al., 1990).

Fibroblast growth factor is a polypeptide of MW 16 kD (Gospodarowicz et al., 1986; Lobb et al., 1986). It is present in a variety of normal tissues including the brain (Lemmon et al., 1982; Gospodarowicz et al., 1984). It is a potent mitogenic for a wide range of neuroectodermal- and mesodermal-derived cells (Gospodarowicz et al., 1986).

Accumulating data suggest that ECM molecules, CAMs and soluble diffusible molecules may not act independently of each other in mediating adhesions. For example, NGF and LN can modulate the expression of N-CAM on PC12 cells (Pollerberg et al., 1986; Prentice et al., 1987; Doherty et al., 1988).

ASTROCYTES AS A SUBSTRATUM FOR CELL CULTURE

A cell culture model is an in vitro system in which individual

cells are grown in an artificial environment. This model comprises basically three parts: cell, substratum, and culture medium. In this system the in vivo environment of cells is mimicked and simplified, and can be changed at will; the morphology and growth behaviour of neurons can be directly observed. It is thus a valuable model for investigating the influences of extrinsic factors on neurons.

Olfactory receptor neurons are a good model for studying neuronal genesis and differentiation. First, they are situated in an epithelium which is easily dissected. Second, they make contact with the CNS directly so that the pathway is simple. And third, they are generated and differentiate continuously throughout life, so ORNs of various developmental stages can be observed and studied.

Purified ECM molecules have been used in culture as substrata to study their effects on neuronal survival and neurite outgrowth, and neurons of different origins respond differently to these molecules. For example, dissociated embryonic chick neurons from dorsal root ganglia extend neurites on both LN and FN, but those from spinal cord and retina only do so on LN (Rogers et al., 1983). A neurite refers to a neuronal process which cannot be identified conclusively as an axon or a dendrite. Previous studies show that ORNs dissociated from adult rat OM cannot extend neurites on non-cellular substrata such as LN, FN and poly-L-lysine (PLL), but are able to do so on cultured astrocytes (Noble et al., 1984b; Chuah et al., 1991).

It has been demonstrated that cultured astrocytes act as an effective substratum for neuronal survival and differentiation. For example, on rat cortical astrocytes, more rat cerebellar and spinal

cord neurons attach to the substratum than on fibroblasts and heart muscle cells. The neurons also survive longer and extend longer neurites on astrocytes (Noble et al., 1984b). It has been suggested that astrocytes promote neuronal growth and differentiation by providing ECM molecules and CAMs on their cell surfaces (e.g. Ard and Bunge, 1988; Drazba and Lemmon, 1989), and secreting promoting factors into the culture medium (e.g. Banker, 1980).

Mature ORNs are identified by three criteria (Farbman, 1988): (a) fully developed dendritic cilia; (b) expression of olfactory marker protein (OMP); and (c) a reasonably long axon. Olfactory marker protein is a cytoplasmic protein of MW 19 kD, and is synthesized by the ORNs at about the time when the olfactory axons make synapses with the OB (Margolis, 1980; Farbman and Margolis, 1980; Miragall and Monti-Graziadei, 1982). A preliminary study by Noble et al (1984a) showed that dissociated ORNs could extend neurites on cortical astrocytes. In his study, he described the ultrastructure of the cell bodies and neurites. Subsequently, Chuah et al (1991) showed that ORNs cultured on astrocytes possessed OMP and growth of their neurites could be inhibited by antibodies to N-CAM, N-cadherin and L1. It is still unknown whether ORN neurite outgrowth on astrocytes can be modified by soluble factors in the culture medium. Furthermore, it would be of interest to investigate the behaviour of ORNs when they are presented with astrocytes from their normal target, the OB. It has been demonstrated that astrocytes from different regions of the brain may have different morphological, functional and antigenic properties. For example, striatal and mesencephalic astrocyte cultures show different

immunoreactivity for GFAP. Dopaminergic neurons from mesencephalon grown on striatal astrocytes have one or a few thin processes while those on mesencephalic astrocytes bear numerous thick ones with varicosities (Denis-Donini et al., 1984).

EXPERIMENTAL PURPOSE AND DESIGN

The purpose of this study is to investigate by using a cell culture model the influence of environmental factors, including surface and soluble molecules, on olfactory neuron differentiation.

Olfactory receptor neurons will be dissociated from the OM and cultured on astrocyte monolayers. Scanning and transmission electron microscopy will be done to verify that ORNs can differentiate morphologically on cultured astrocytes.

In addition to cortical astrocytes, glial cells will be purified from the OB and the ONL. Fibroblasts will also be used to determine if there is any difference when ORNs are grown on a non-glial substratum. Fibroblasts are chosen because they are present in the lamina propria and contact olfactory axons while the latter course towards the OB. Furthermore, fibroblasts are easy to culture and large quantities of these cells may be produced in vitro. Olfactory neurite growth on various substrata will be measured by quantifying the percentage of ORNs bearing neurites and the neurite length.

To address the question of whether soluble factors in the culture medium play a role in regulating neurite outgrowth, ORNs will be cultured on cellular substrata in the presence of medium conditioned by cells other than those forming the substrata.

MATERIALS AND METHODS

PREPARATION OF CELLULAR SUBSTRATA

(1) Cortical Astrocyte Monolayers

Cortical astrocytes were prepared by a method modified from that of McCarthy and deVellis (1980). Newborn (1-3 day-old) Sprague-Dawley rats were decapitated and the cerebral cortices were isolated aseptically. The cortices were dissected free of meninges in Hank's balanced salt solution (HBSS) (Sigma, St. Louis, U.S.A.), and cut into pieces small enough to pass through a fire-polished Pasteur pipette. The pieces were then incubated in HBSS containing 0.125% trypsin (Sigma) (3 ml of HBSS plus 3 ml of 0.25% trypsin in HBSS for cortices from 3 rats) at 37°C for 25 minutes. Trypsinization was stopped by removing as much as possible the trypsin-containing HBSS, and adding fresh HBSS (2 ml) containing trypsin inhibitor (Sigma, catalogue number: T9128) and deoxyribonuclease (DNAase) (Sigma) at 50 μg/ml and 40 μg/ml respectively. The tissue was triturated through a wide-bore flamed Pasteur pipette which was about 1.5 mm in diameter, and the released cells were collected by filtration through a 72-μm nylon mesh and centrifugation at 500g for 10 minutes. The resulting cell pellet was resuspended in the culture medium which is Dulbecco's modified Eagle's medium (DMEM) (Gibco, New York, U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 1% MEM-vitamin solution (Gibco), and 1% penicillin and streptomycin (Sigma). The final concentrations of penicillin and streptomycin were 100 units/ml and 100 mg/ml respectively. This culture medium is referred to as CNS medium in the rest of the text. Viability of the cells was evaluated by staining a

cell sample with 0.2% trypan blue and counting the dye-exclusive cells with a hemacytometer. The cells were plated at about 1-2 x 10^7 cells per 75-cm^2 flask (Falcon, New Jersey, U.S.A.), and maintained in 10 ml of CNS medium at 37°C in a humidified atmosphere of 5% CO^2 and 95% air. Prior to cell plating the flasks were coated with PLL (Sigma) by overnight incubation in 5 µg/ml PLL in distilled water, followed by washing in distilled water, and air-drying.

After 10 days' culture, the flasks were washed twice with DMEM and shaken on a rotary shaker overnight in DMEM at 37°C and 140rpm to remove the remaining neurons and oligodendrocytes. On the following day, flasks were washed and 10 ml of HBSS were added to each of them. Next about 10 drops of 0.25% trypsin in HBSS were added. The flasks were incubated at 37°C, and checked periodically on a Nikon phase contrast microscope until the cells rounded up, when 2 ml of CNS medium was added to terminate the trypsinization. The cells were triturated with a fire-polished Pasteur pipette, centrifuged at 500g for 10 minutes, and resuspended in CNS medium. The cell suspension was aliquoted into 2 or 3 PLL-coated flasks.

The cells were allowed to attach for 24 hours, and 10^{-7}M cytosine arabinoside (Ara-C) (Sigma) was added to kill the rapidly dividing fibroblasts. After 2 days' incubation, the culture medium was changed and again supplemented with 10^{-7}M Ara-C. This was repeated one more time and subsequently the cultures were fed with fresh CNS medium every 3-4 days for 2 weeks, and thereafter once a week. The purified astrocytes usually became confluent and could be replated onto coverslips 1 week after the last Ara-C treatment.

To obtain monolayers of astrocytes, the flask was washed with 3 changes of HBSS while saving the conditioned medium. About 6-7 drops of 0.25% trypsin in HBSS was added, and the flask was incubated at 37°C until the astrocytes rounded up. Trypsinization was stopped by adding about 2 ml of CNS medium, and the astrocytes were triturated, centrifuged and resuspended in conditioned medium. The cells were plated at a concentration of 10^4 cells per PLL-coated 12-mm round coverslips placed in 24-well plates (Nunclon, Denmark), and 4 x 10^4 cells per PLL-coated 35-mm petri dishes (Falcon). The cells were incubated for at least 2 hours to let them adhere, then a 1:1 mixture of conditioned medium and fresh CNS medium was added. The monolayers were maintained by weekly feeding with fresh CNS medium.

The cortical astrocyte monolayers used in the experiments were 95-98% pure as judged by immunofluorescent staining for GFAP (Fig. 1A). The astrocytes were used within 2 months of culture.

(2) OB Astrocyte Monolayers

The OB astrocytes refer to those present in all layers of the OB excluding the ONL. Newborn Sprague-Dawley rats were also used. In order to verify the clean removal of ONL from the OB, random pieces of these two parts were processed for light microscopy.

They were fixed in Bouin's fixative for about 5 hours, and dehydrated through 70%, 80%, 2 changes of 90%, and 3 changes of 100% alcohol for 30 minutes each. The alcohol was replaced by 2 changes of xylene, which was substituted by 3 changes of paraffin, each for 30 minutes. The specimens were finally embedded in fresh paraffin, and cut into 5 μm thick sections with a Reichert-Jung microtome. The

paraffin sections were deparaffinized in xylene, hydrated in decreasing concentrations of alcohol down to 70%, and stained with hematoxylin and eosin (H & E). Then the sections were dehydrated through graded alcohol, cleared in xylene, and mounted in Permount (Fisher Scientific, New Jersey, U.S.A.).

Examination of the sections on a Nikon light microscope showed that the peeled part was separated from the remaining part at the boundary between ONL and GLM. The ONL was peeled with the meninges (Fig. 2).

The procedure for purifying OB astrocytes was similar to that for cortical astrocytes. About 5 x 10^6 cells could be obtained from 5 rats, and the cells were plated in a 25-cm^2 flask (Corning, New York, U.S.A.). The OB astrocytes used were 93-97% GFAP-positive (Fig. 1B). They were also used within 2 months of culture.

(3) ONL Glial Cell Monolayers

The ONL glial cells were prepared by a method modified from that of Brockes et al (1979), and that of Assouline et al (1989). The ONL was dissected in minimum essential medium Eagle with 25 mM HEPES (MEM-H) (Sigma), and then incubated in 3 ml of MEM-H containing 0.03% collagenase (type III) (Sigma) and 0.125% trypsin at 37°C for 15 minutes. The supernatant was removed as much as possible, and the ONL was treated with fresh collagenase in MEM-H in the same way for another two times. Then 2 ml of the supernatant was removed and 1 ml of CNS medium was added. The tissue was triturated through a flamed Pasteur pippete, and the dissociated cells were filtered through a 72-μm nylon mesh, and centrifuged at 500g for 10 minutes. The cells were

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resuspended in CNS medium, and plated in a 25-cm^2 flask. About 2 x 10^6 cells were derived from 10 rats.

The cells were allowed to attach for 24-48 hours, then Ara-C was added at 10^{-7}M for 3 days. The flask was washed with 3 changes of fresh CNS medium within an hour, and bovine pituitary extract (Sigma) was added at 100 µg/ml in the last change to stimulate division of ensheathing cells. After 3-4 days, the cells were fed with fresh CNS medium supplemented with bovine pituitary extract for a further 3-4 days. Next the flask was rinsed with 3 changes of HBSS, and the cells were trypsinized, triturated, and pelleted. The cells were resuspended at 250 µl of monoclonal mouse anti-Thy-1.1 IgG (Serotec, Oxford, England) diluted 1:500 in CNS medium per 10^6 cells, and incubated at 37°C for 30 minutes. Thy-1.1 is a small glycoprotein found on fibroblasts. The cells were then centrifuged. The pellet was washed once in CNS medium, then resuspended in 3 ml of CNS medium. The cell suspension was transferred to a 25-cm^2 flask coated with goat anti-mouse IgG (Sigma), and incubated at 4°C for 60 minutes. The coating of the antibody was prepared by incubating the flask in 3 ml of goat anti-mouse IgG in 0.135M NaCl at 1 mg/ml at room temperature (RT) for 1 hour, and then at 4°C for 12-18 hours. The flask was washed with 0.01M phosphate buffered saline (PBS) (1.15 g of Na_2HPO_4 plus 8 g of NaCl, 0.2 g of KCl and 0.2 g of KH_2PO_4 in 1000 ml of distilled water, pH 7.4) prior to use. The fibroblasts would adhere to the flask while the nonadherent cells were collected and centrifuged. The cells were resuspended in CNS medium supplemented with bovine pituitary extract, and plated in 25-cm^2 flasks. Culture medium was changed

every 4 days. Prior to plating on coverslips, the cells were purified a second time with anti-Thy-1.1.

The ensheathing cells used in the experiments were 73% pure as determined by their morphology and their positive staining for S100 protein (Fig. 1C, D). S100 protein is a family of small acidic cytosol proteins that is generally accepted as a marker for Schwann cells and astrocytes.

(4) Skin Fibroblast Monolayers

A piece of skin was dissected from the back of a newborn Sprague-Dawley rat. The skin was cut into small pieces of about 1 mm^3 in HBSS on a dissecting microscope with the hypodermis facing the surface of the petri dish. Then 4-5 explants of skin were transferred to each 35-mm petri dish, and CNS medium was added such that the hypodermis remained attached to the petri dishes. During the next 3 days fusiform cells grew out of the explants on the surface of the petri dishes, and more culture medium was subsequently added to cover the explants completely. When the cultures had become confluent about 10 days after plating, the explants were discarded. The remaining cells in the dishes were trypsinized in HBSS, triturated, and centrifuged at 500g for 5 minutes. The pellet was resuspended in CNS medium and plated onto flasks at about 2×10^5 cells per 25-cm^2 flask. The cells were passaged about once a week, and were used within 10 passages.

Before being plated onto coverslips, the cells were incubated in 10 μg/ml of Mitomycin-C (Sigma) in fresh CNS medium for 4 hours to inhibit fibroblast division. The flask was then washed with HBSS and

the cells were trypsinized, triturated, and centrifuged at 500g for 5 minutes. The pellet was resuspended in CNS medium and plated in a drop onto 12-mm round coverslips at 10^4 cells per coverslip. The cells were allowed to attach for 2 hours, and then more CNS medium was added.

The fibroblast monolayers prepared in this way were stained for fibronectin, a protein secreted by fibroblasts, with VECTASTAIN ABC Kit (Vector, California, U.S.A.). Judged by the positive immunoreaction, the monolayers used in the experiments were 95-97% pure (Fig. 1F).

DISSOCIATION OF OLFACTORY MUCOSA

The OM was dissociated by the method of Chuah et al (1991). A 4-5 week-old Sprague-Dawley rat was killed with an overdose of ether and decapitated. The nasal septum was isolated aseptically and transferred to HBSS in which the OM was dissected. The OM was cut to small pieces, and incubated in 0.08% trypsin in HBSS at 37°C for 20 minutes. Then the trypsin-containing HBSS was removed, and the tissue was trypsinized in the same condition for another 20 minutes. The solution was again removed, and trypsin inhibitor and DNAase in HBSS (1.5 ml) was added at 50 μg/ml and 40 μg/ml to terminate the trypsinization. The tissue was triturated through a wide-bore flamed Pasteur pipette, and the dissociated cells were harvested by filtration through a 72-μm nylon mesh. Next the cells were centrifuged at 500g for 10 minutes, and resuspended in CNS medium. About 1.2×10^4 cells were plated onto each 12-mm round coverslips placed in 24-well plates, and about 4×10^4 cells onto a 35-mm petri dish. Confluent cellular substrates had been prepared on the coverslips and petri dishes prior to the addition of the ORNs. The ORNs were grown for 2 days, then were processed for

electron and fluorescent microscopy.

TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

Cells were cultured on 35-mm petri dishes for transmission electron microscopy, and on both 35-mm petri dishes and 12-mm coverslips for scanning electron microscopy. The cultures were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (405 ml of 0.2M Na_2HPO_4 plus 95 ml of 0.2M NaH_2PO_4, pH 7.4) at 4°C for 10 minutes, and rinsed with 3 changes of 0.1M Sorensen's phosphate buffer (3.8 ml of 0.5M NaH_2PO_4 plus 16.2 ml of 0.5M Na_2HPO_4, in 4 times in volume of distilled water, pH 7.4) at 4°C for 5 minutes each. The cultures were postfixed with 0.1% osmium tetroxide at RT for about 20 minutes, and washed with 2 changes of 0.1M Sorensen's phosphate buffer for 5 minutes each. The cultures were then dehydrated through 50%, 70%, 85%, 95%, and 3 changes of 100% alcohol for 10 minutes each. Cultures in petri dishes were detached as a layer from the petri dishes with propylene oxide, then washed free of propylene oxide with absolute alcohol.

For transmission electron microscopy, cultures were processed in 2:1 and 1:1 mixtures of absolute alcohol and Spurr for 2 hours and 1 hour respectively, then in pure Spurr overnight. Spurr is a mixture of 26g of noneyl succinic anhydride (Emscope, Kent, England), 6g of DER 736 resin (Electron Microscopy Sciences, Pennsylvania, U.S.A.), 10g of vinyl cyclohexene dioxide (Electron Microscopy Sciences), and 0.4g of 2-dimethylaminoethanol (Polysciences, Pennsylvania, U.S.A.). On the following day, the cultures were transferred to fresh pure Spurr for about 1 hour before embedding in fresh Spurr in an oven set at 70°C.

Ultrathin sections were made with a Reichert-Jung ultramicrotome, and mounted on grids with 200 meshes. The sections were stained with 1% aqueous uranyl acetate solution in the dark for 10 minutes, and rinsed in 3 changes of distilled water. Then the sections were stained with lead citrate for 10 minutes in a sealed container with sodium hydroxide placed inside. The grids were rinsed in 3 changes of distilled water and dried. The specimens were viewed on a JEOL JEM-100CXII TEM at 80 kV.

For scanning electron microscopy, after fixation and dehydration, cultures were processed through 20%, 40%, 60%, 80% Freon 113 in absolute alcohol and 2 changes of 100% Freon 113 for 10 minutes respectively. Then the cultures were critical-point-dried with a LOAD critical point dryer, and coated with gold with an Edwards sputter coater. The specimens were viewed in a JEOL JSM-35CF SEM at 15 kV.

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Cultures on 12-mm round coverslips were fixed with 4% paraformaldehyde in 0.1M phosphate buffer at RT for 15 minutes, and rinsed with 4 changes of MEM-H. The coverslips were then incubated in acid alcohol (95 ml of absolute alcohol plus 5 ml of glacial acetic acid) at -4°C for at least 15 minutes, and again rinsed with MEM-H. The primary antibody was applied at 40 µl per coverslip, and was incubated at RT for 30 minutes. The coverslips were rinsed in MEM-H, and the secondary antibody which was conjugated with fluorescein isothiocyanate (FITC) was applied in the same way. The coverslips were washed in MEM-H, and mounted on glass slides. The mounting media for fluorescent microscopy consisted of 400 µg/ml of phenylenediamine

(Sigma) in 0.1M sodium carbonate solution plus 4-8 times in volume of glycerol. The cultures were examined on a Nikon fluorescent microscope.

The above method was used to stain for GFAP, neurofilaments, S100 protein and Thy-1.1. The antibodies used are listed in Table 1. All antibodies and blocking serum were diluted 1:100 in MEM-H.

The neural cell adhesion molecule was stained in a similar way. The exceptions were: (1) Antibody to N-CAM was diluted 1:200. (2) The cells were not fixed until the primary and secondary antibodies had been applied. The antibodies used are shown in Table 1.

N-cadherin was stained with the method of Hirano et al (1987). The coverslips containing astrocytes or fibroblasts were washed in HEPES-buffered Ca^{2+}- and Mg^{2+}-free saline (0.85% NaCl with 10 mM HEPES) supplemented with 1 mM $CaCl_2$ (HMF), then fixed in 3.5% paraformaldehyde in HMF at 4°C for 30 minutes. The fixative was washed away in 50 mM Tris-buffered saline (TBS) (900 ml of 0.85% NaCl plus 100 ml of 0.05 M Tris buffer (Sigma), pH 7.4) containing 1 mM $CaCl_2$ (TBS-Ca), and acetone was applied to the coverslips at -4°C for 10 minutes. After rinsing the coverslips in TBS-Ca, 1% bovine serum albumin (BSA) (Sigma) in HMF were applied at 40 µl/coverslip at RT for 30 minutes. The serum was blotted, and mouse monoclonal anti-N-cadherin antibody (Sigma) diluted 1:100 in HMF was added at RT for 60 minutes. The coverslips were washed in TBS-Ca, then a FITC-conjugated goat anti-mouse IgG (Jackson, Pennsylvania, U.S.A.) diluted 1:100 in HMF containing 1% BSA was applied at RT for 60 minutes. The coverslips were washed in TBS-Ca and then distilled

water, and finally mounted in the mounting media for fluorescent microscopy.

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VECTASTAIN ABC Kit (Vector) was used to stain for fibronectin on fibroblasts. Fibroblast monolayers on 12-mm round coverslips were fixed in 4% paraformaldehyde in 0.1M phosphate buffer at RT for 15 minutes, and then rinsed for 5 minutes in 0.1M phosphate buffer. The coverslips were incubated at 37°C for 20 minutes in normal goat serum diluted 1:70 in 0.3% Triton X-100 (Sigma) in PBS. The excess serum was blotted. The primary antibody, mouse monoclonal anti-fibronectin antibody (Sigma), was diluted 1:600 in 0.1M phosphate buffer and applied at 40 µl per coverslip for 30 minutes. The cultures were rinsed for 10 minutes in buffer, then incubated at 37°C for 30 minutes with diluted biotinylated goat anti-mouse IgG solution. The cultures were rinsed again in buffer for 10 minutes, and incubated with VECTASTAIN avidin-biotin-horseradish peroxidase (ABC) complex at 37°C for 60 minutes. Next the coverslips were washed in buffer for 10 minutes. Final reaction product was visualized by incubating the coverslips at RT for 7 minutes in 0.1M Tris buffer (pH 7.2) containing 0.1% diaminobenzidine tetrahydrochloride (Sigma) and 0.015% hydrogen peroxide. The coverslips were washed free of the substrate solution in tap water for 5 minutes. The cultures were then counterstained with H & E, and mounted in Permount.

For the control, the primary antibody was substituted with buffer.

COUNTING OF ORNs BEARING NEURITES AND MEASUREMENT OF NEURITE LENGTH

The ORNs on the cellular substrata were labelled with

anti-neurofilament antibody (RT97), and the number of ORNs bearing neurites were counted. The ORNs bearing neurites referred to those with at least one process greater than 2 cell bodies long. Only single neurons were counted, those in clumps were not included.

The lengths of the neurites were also measured. The ORNs with neurites were randomly chosen and drawn on graph paper with the use of a graticule fitted in the objective of the microscope. The graticule was marked with squares of 0.5 mm wide, and was calibrated so that 0.5 mm equalled 10 μm. The longer neurite of each ORN was measured by using a SAC (Science Accessories Corporation) digitizer connected to an IBM computer.

ANALYSIS OF CONDITIONED MEDIA

(1) Preparation of Conditioned Media

Conditioned media (CM) of cortical astrocytes (CMA) and fibroblasts (CMF) were media conditioned by cortical astrocytes or fibroblasts after 5 day's culture in CNS medium. To exclude possible contamination of cells in the conditioned media, the media were frozen at -70°C for at least 1 hour, then thawed and centrifuged at 500g for 10 minutes prior to use.

(2) Ultrafiltration

To divide the conditioned media into fractions containing molecules of different MW ranges, the conditioned media were filtered with an Amicon stirred ultrafiltration cell (Model 8010). The membrane ultrafilters (Amicon's Diaflo) used were of YM-type which was hydrophilic and had exceptionally low non-specific protein binding. The membranes used had MW cutoffs of 3,000, 10,000 and 30,000 daltons

respectively, which meant they allowed only molecules of MW <3,000, 10,000 and 30,000 daltons to pass through. Sterilized stirred ultrafiltration cell and membrane were assembled aseptically and about 10 ml of CM was added into the cell. The cell was connected to a nitrogen pressure source and operated at a setting under 75 psi (5.3 kg/cm^2). The filtration was carried out on a magnetic stirring table and normally lasted around 45 minutes. About 0.2 ml of CM was retained each time. The filtrate was analysed by culturing the ORNs with the filtrate as culture media to examine the effects of the filtrate on ORN neurite extension.

(3) Heat Treatment

Conditioned media of cortical astrocytes and fibroblasts were boiled at 100°C for 5 minutes. The CNS medium was treated in the same way to serve as a control.

(4) Protein Assay

The protein content of CMA, CMF and CNS medium was determined by Bio-Rad protein assay. The procedure was according to instructions in the kit. Standards containing 0-1.4 mg/ml of lyophilized bovine serum albumin were prepared with HBSS. Samples of CMA, CMF and CNS medium were diluted 1:8 in HBSS. Then 0.1 ml of the standards and samples of CMA, CMF and CNS media were mixed with 5 ml of diluted dye reagent respectively. The optical densities (OD) of the standards and samples versus reagent blank at 595 nm were measured with a LKB spectrophotometer. Standard curve was plotted with OD_{595} of the standards measured versus concentration, and the protein content of the samples was read from the standard curve.

(5) Investigation of Concentration-Activity Relationship

Conditioned medium of fibroblasts was frozen, thawed and centrifuged as mentioned above. Then 30 ml of CMF was filtered through a membrane ultrafilter with a MW cutoff at 3,000 daltons, and the volume concentrated down to 1 ml. Next 2 ml of DMEM was added into the cell and stirred on a magnetic stirring table for 3 hours to wash the membrane ultrafilter. The 3 ml of 10 times concentrated CMF containing molecules of MW >3,000 daltons was diluted with DMEM sequentially to get 5, 2.5 and 1 times concentrated CMF. The concentrated CMF was used to culture ORNs, and the percentage of ORNs bearing neurites as well as the mean number of ORNs attached to astrocytes were examined. Concentration curves were plotted versus the number of times CMF was concentrated.

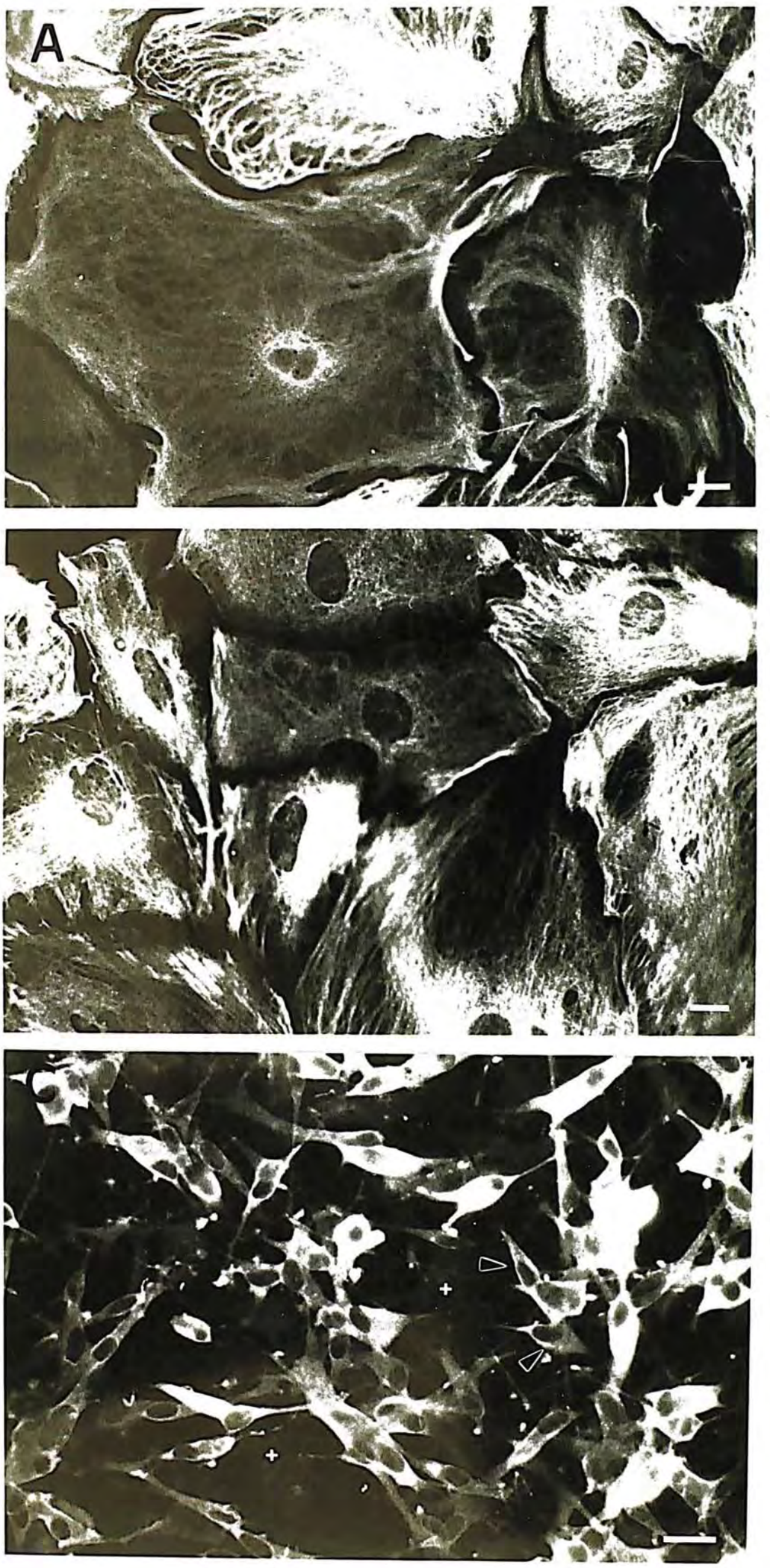
Table 1. List of Antibodies for Immunofluorescence.

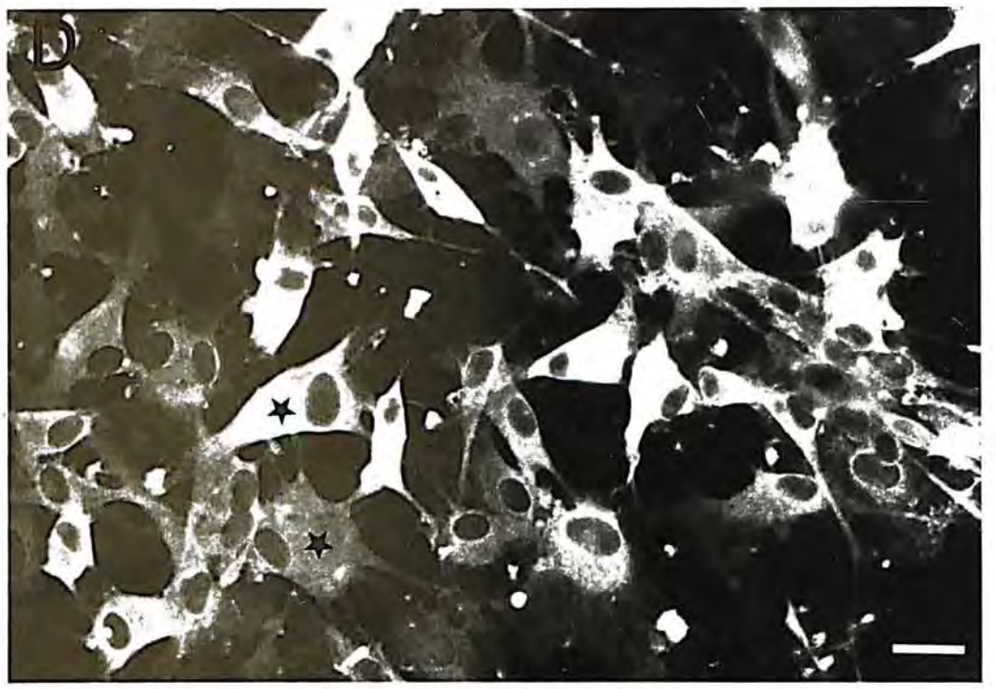
Antigens stained	Primary Antibody: Experimental specimens	Primary Antibody: Control	Secondary Antibody
GFAP	R anti-GFAP Ab (polyclonal) NGS	NGS	G anti-R-FITC NGS
Neurofilaments	M anti-neurofilament Ab (RT97) (monoclonal) NGS	MEM-H	G anti-M-FITC NGS
S100 Protein	R anti-S100 Protein Ab (polyclonal) NGS	NGS	G anti-R-FITC NGS
Thy-1.1	M anti-Thy-1.1 Ab (monoclonal) NGS	MEM-H	G anti-M-FITC NGS
N-CAM	M anti-N-CAM Ab (monoclonal) NGS	MEM-H	G anti-M-FITC NGS

R: rabbit; G: goat; M: mouse; NRS: normal rabbit serum; NGS: normal goat serum; Ab: antibody. NGS and NRS are from Vector, G anti-R-FITC and G anti-M-FITC from Jackson. R anti-GFAP Ab is from Sigma, R anti-S100 protein Ab from Dako (Denmark), M anti-Thy-1.1 Ab and M anti-N-CAM Ab from Serotec. RT97 was a generous gift from Dr. S. David, Montreal General Hospital Research Institute.

Fig. 1. Micrographs of various cellular substrata. (A) Cortical astrocytes in a monolayer stained for GFAP. The cells are polygonal in shape. The distribution of astroglial filaments is uneven. Bar=20 μm. (B) OB astrocyte monolayer stained for GFAP. The cells are similar to cortical astrocytes in morphology. Bar=20 μm. (C) ONL glial cells labelled with S100 protein. The top and more heavily stained cells are the ensheathing cells. The typical ones are fusiform-shaped with slender processes (arrowheads). Note that the ensheathing cells are not fully confluent. The flat and bigger cells at the bottom are less heavily labelled, and are probably cortical astrocytes (crosses). These cells are not clearly shown in this picture because they are at a different focal depth. Bar=20 μm. (D) This picture shows the ensheathing cells which are flatter, some of which extend more than two fine processes from their cell bodies (asterisks). Bar=20 μm. (E) Phase contrast micrograph of fibroblasts in a culture flask. The cells are flat and irregular in shape. Bar=100 μm. (F) Fibroblast monolayer immunostained for fibronectin. The arrows indicate the reaction product. Bar=20 μm.

Fig.1

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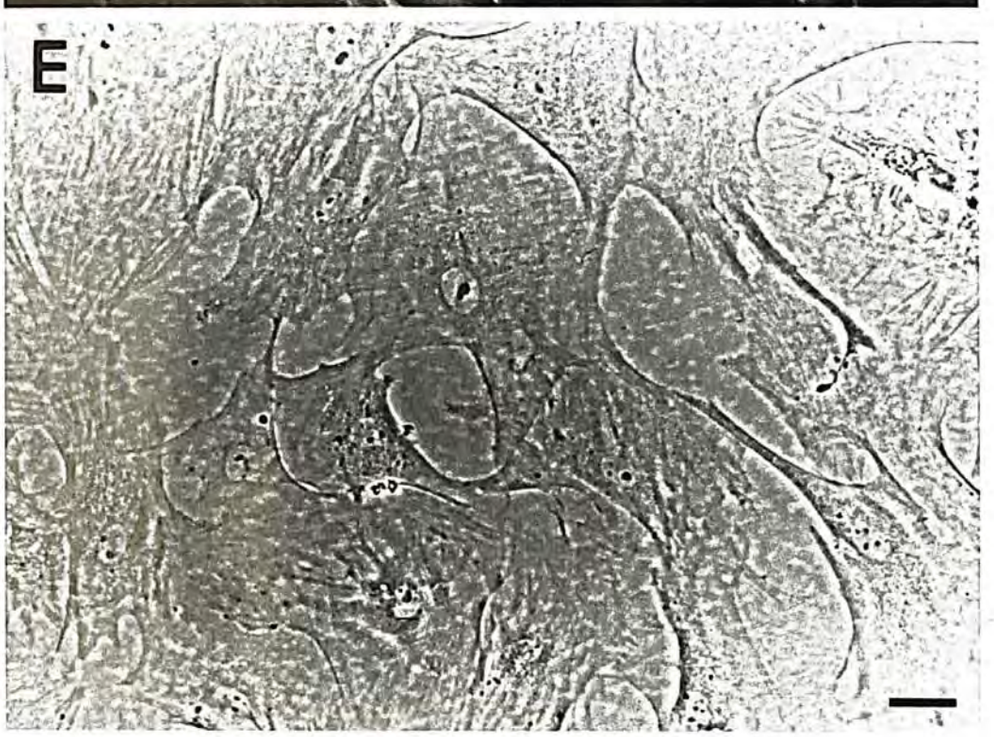
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F

Fig. 2. Light micrograghs of ONL (A), and the remaining OB (B). (A) The ONL consists of numerous nerve bundles. Bar=100 μm. (B) The layers of the remaining OB: 1. granule cell layer; 2. internal plexiform layer, which is not obvious in newborn rats; 3. mitral cell layer; 4. external plexiform layer; and 5. glomerular layer. Bar=100 μm.

Fig. 2

A

B

RESULTS

TRANSMISSION AND SCANNING ELECTRON MICROSCOPY

(1) Cortical Astrocytes

Cultured cortical astrocytes were processed for transmission and scanning electron microscopy to examine their morphology under culture conditions. It was observed on SEM that the astrocytes were flat and mainly polygonal in shape, although some cells extended processes. The astrocytes had various modifications on their surfaces. Some had numerous round or microvillous-like protrusions of different lengths extending from the cell surfaces, while others appeared smooth (Fig. 3). Some cells had only uneven or smooth surfaces, while some had both. There was no preferential distribution of the two types of surfaces. Both might be present on the peripheral as well as on the central areas of the cells. Junctions between astrocytes were composed of either relatively smooth (Fig. 3A) or more jagged, irregular cell edges (Fig. 3B). Occasionally astrocytes extended processes which overlapped adjacent cells (Fig. 3C). Interdigitation of finger-like processes between adjacent cells were also observed (Fig. 3D).

On TEM, similar surface characteristics of astrocytes as those observed on SEM were present. Finger-like and round protrusions were seen extending from some surfaces of astrocytes (Fig. 4A). Most astrocytic surfaces observed were smooth (Fig. 4B). Astroglial filaments were present in bundles or scattered in the cytoplasm (Fig.4). As observed on SEM, adjacent astrocytes sometimes overlapped each other (Fig. 4C, D), and fuzzy material might be present at the contact sites (Fig. 4C).

(2) ORNs on Cortical Astrocytes

Electron microscopy of ORNs was undertaken after culturing them on cortical astrocyte monolayers for 2 days. On SEM bipolar cells which resembled ORNs were observed. The cells had pear-shaped cell bodies of about 10 μm in diameter and their surfaces were usually rough (Fig. 5A). The thicker and shorter process, presumably the dendrite, ended in a swelling from which fine filopodia extended (Fig. 5B, C). The other process, presumably the axon, was thinner and longer. It was about 0.2 μm in diameter and had varicosities along its length (Fig. 5B, D). No fasciculation of these processes were observed. There was no obvious preferential distribution of the ORNs on either the smooth astrocytic surface or that with numerous protrusions.

Verification that the long, thin processes of the ORNs were axons was conducted by transmission electron microscopy. The axons were characterized by the presence of microtubules in their cytoplasm. It appeared that more axons were present on the smooth astrocytic surface (Fig. 6A), and fewer were in contact with finger-like protrusions (Fig. 6B), although no quantitative study was done to confirm this. Observation of the ORNs on TEM showed that they had big, round nuclei containing clumps of heterochromatin. Coated vesicles were sometimes present on the contact sites between the cell bodies and the astrocytic surfaces (Fig. 6C).

The SEM and TEM observations that the ultrastructure of the ORNs is similar to that <u>in vivo</u>, support the notion that ORNs can differentiate morphologically on cultured astrocytes. The present data confirm and extend findings from previous studies (Noble et al., 1984a;

Chuah et al., 1991) which have shown that ORNs are bipolar and may be ciliated when cultured on cortical astrocytes. Moreover, the presence of coated vesicles between astrocytes and ORN cell bodies is indicative of interaction between these two cell types. This interaction may be crucial for the growth of axonal processes as has been found in cerebellar neurons of neonatal and adult rats (Altman, 1971), and neuronal growth cones of grasshopper embryos (Bastiani and Goodman, 1984).

OLFACTORY NEURITE EXTENSION ON CORTICAL ASTROCYTES, OB ASTROCYTES, ONL GLIAL CELLS, AND FIBROBLASTS

(1) Morphology of the Cellular Substrata

Cortical astrocytes were stained for GFAP and observed under a fluorescent microscope (Fig. 1A). In a confluent monolayer the cells were flat and polygonal in shape. Cells at the periphery of the monolayer tended to be larger in size, while those in the center, because of the higher density, were smaller and occasionally overlapped each other.

Olfactory bulb astrocytes refer to those present in all layers of the OB excluding the ONL. The cells were also stained for GFAP and examined under a fluorescent microscope (Fig. 1B). They were similar in morphology to cortical astrocytes.

In vivo the ONL glia consists of ensheathing cells and astrocytes (Doucette, 1984, 1989). The purified ONL glial cells in the experiments were stained for S100 protein, a protein present on Schwann cells and astrocytes, and examined under a fluorescent microscope. Two types of S100 protein-positive cells were observed. One type was the

ensheathing cells. They were heavily stained with S100 protein, and were located at the top of the cultures (Fig. 1C, D). Typical ensheathing cells were fusiform-shaped with slender processes, while some appeared more flattened and had more than two processes emanating from the cell body. Because the cell bodies of the ensheathing cells were comparatively smaller in size than astrocytes from cerebral cortex and OB, it was extremely difficult to obtain a confluent layer. The ONL glial cell cultures used were of 73% ensheathing cells. The other type of S100 protein-positive cells was less intensely labelled, and formed a separate layer underneath the ensheathing cells (Fig. 1C). They were flat, and much bigger than the ensheathing cells. These cells were not fibroblasts because immunohistochemical staining for Thy-1.1, a small glycoprotein normally found on fibroblasts, resulted in only 2-3 cells being labelled per coverslip (not shown). As the ONL is known to contain ensheathing cells and astrocytes in vivo, most likely these underlying cells were astrocytes.

Under the phase contrast microscope, fibroblasts in primary cultures were fusiform-shaped and oriented parallel to one another (not shown). After being subcultured once or twice, the fibroblasts became flat and irregular in shape (Fig. 1E). They were immunostained positively for fibronectin (Fig. 1F).

(2) Morphology of ORNs on Various Cellular Substrata

Olfactory mucosa was dissociated and the resulting cells plated onto various cellular substrata. The antiserum RT97 was used to distinguish ORNs from the other cell types.

The morphology of ORNs was similar on cortical astrocytes, OB

astrocytes and ONL glial cells. A percentage of ORNs extended neurites on these substrata. As the dendrites had few or no neurofilaments, most of them were not stained and the ORNs often appeared unipolar (Fig. 7A-F). The ORN cell bodies were pear-shaped, but some cell bodies, especially those bearing no neurites, looked round. The axons were long, and had many varicosities and bifurcations along their courses (Fig. 7A-D, F). Some axons could be seen ending in a flat dilatation (Fig. 7B).

On fibroblast monolayers, there were notably fewer ORNs which attached to the substratum, and there were also fewer axons. The axons were generally shorter, and had less bifurcations compared to those cultured on the other substrata (Fig. 7E). The varicosities of the axons were also less prominent so that the axons looked slender.

(3) Quantitative Studies of Olfactory Neurite Extension on the Cellular Substrata

To investigate quantitatively the effects of the cellular substrata in supporting ORN differentiation, the following parameters were measured: (1) percentage of ORNs bearing neurites, (2) average neurite length. The mean number of ORNs attached to the substrata per coverslip was also examined. The results are summarized in Tables 2 and 3.

For cortical and OB astrocytes, the number of ORNs attached to the substrata, percentage of cells bearing neurites, and average neurite length were not significantly different among the two groups (Table 2). On fibroblasts, however, there were significantly less ORNs attached, and fewer cells with neurites. The neurites were also

significantly shorter.

The ONL glial cells were maintained in CNS medium supplemented with bovine pituitary extract at 100 μg/ml. Hence, as a control, ORNs were also cultured on cortical astrocytes in CNS medium supplemented with bovine pituitary extract (Table 3). The ORNs in these two groups did not differ significantly in terms of percentage of cells with neurites, neurite length, and the mean number of ORNs attached per coverslip.

The morphological and quantitative results suggest that cortical astrocytes, OB astrocytes and ONL glial cells are similarly effective substrata for olfactory neurite extension. In contrast, fibroblasts are much less effective in supporting ORN attachment and subsequent extension of neurites. The overall poorer rate of differentiation observed on fibroblasts may be mediated by two factors: (1) cell surface interactions between ORNs and fibroblasts, and (2) soluble substances secreted by the fibroblasts into the culture medium.

CELL ADHESION MOLECULES ON ASTROCYTES

It has been shown that neurite extension of ORNs on cortical astrocytes is inhibited by antibodies to cell adhesion molecules such as N-CAM, L1 and N-cadherin (Chuah et al., 1991). By immunofluorescence, N-CAM and N-cadherin were shown to be present throughout the cell surface of astrocytes (Fig. 8A, B). The N-CAM staining was in a punctate pattern and more prominent on the edges of the astrocytes. Olfactory receptor neurons were also N-CAM-positive (Fig. 8C). Fibroblasts expressed N-cadherin (Fig. 8D,E) but not N-CAM (not shown). These results confirm that ORN neurite extension on

cortical astrocytes is mediated by N-CAM and N-cadherin which are expressed on the cell surface. The N-cadherin on fibroblasts may account in part for the small percentage of ORNs which do extend neurites on this substratum.

Until now there are no published data concerning the effects of soluble substances in mediating olfactory neurite extension. The following experiments were conducted to elucidate any possible regulatory role of soluble factors in this aspect of differentiation.

INFLUENCE OF SOLUBLE SUBSTANCES ON OLFACTORY NEURITE EXTENSION

To investigate the role of soluble substances secreted into the culture media by the cellular substrata in mediating olfactory neurite extension, CMA and CMF were prepared and used as culture media. As large amounts of cortical astrocytes could be purified easily from the neonatal cortex, they were used in this part of the project.

The ORNs were cultured either on cortical astrocytes with CMF, or on fibroblasts with CMA for 2 days, then labelled with RT97. Morphology of the ORNs, the percentage of ORNs bearing neurites, and the mean number of ORNs attached to the substrata were examined on a fluorescent microscope. The results are summarized in Table 4.

It was observed that the pattern of the ORNs cultured on astrocytes in the presence of CMF was similar to those cultured in CNS medium. The mean number of ORNs attached to astrocytes was also not significantly changed, whereas the percentage of ORNs having neurites was reduced significantly from 66% to 55%.

When the ORNs were grown on fibroblasts in the presence of CMA, the morphology of the ORNs were also similar to those cultured in CNS

medium. However, significantly more ORNs attached to fibroblasts as compared with those cultured in CNS medium. The percentage of ORNs bearing neurites was not significantly changed.

Reduced olfactory neurite extension in the presence of CMF suggests that fibroblasts may be secreting certain inhibitory substance(s). In view of the fact that CMA does not enhance neurite extension on fibroblasts, it can be deduced that surface interaction is largely responsible for neurite extension on astrocytes. On the other hand, soluble factor(s) in CMA is found to facilitate cell attachment.

Several reports show that soluble substances in conditioned media act by adsorbing to culture substrata (e.g. Collins, 1980; Lander, et al., 1982). To investigate whether the fibroblastic secretion(s) in CMF acts by adsorbing to the cortical astrocytes or is present in soluble form, astrocytes were first treated with CMF for about 6 hours. Then the CMF was changed to CNS medium, and ORNs plated onto the astrocytes. The ORNs were cultured for 2 days, then labelled with RT97 and examined on a fluorescent microscope as in the last experiment. The results are summarized in Table 5. It was found that the percentage of ORNs bearing neurites was significantly decreased from 68% to 63%. The ORNs also extended significantly shorter neurites, whereas the number of ORNs attached to the astrocytes per coverslip was not significantly changed. The results suggest that the inhibitory soluble substance(s) in CMF adsorbs to the substratum, thereby inhibiting olfactory neurite extension.

To investigate the nature of the inhibitory substance(s) in CMF, the following experiments were done.

ANALYSIS OF MEDIA CONDITIONED BY FIBROBLASTS

(1) Molecular Weight Range of the Inhibitory Substance(s)

To determine the MW range of the soluble inhibitory substance(s), CMF was divided into fractions of different MW ranges by ultrafiltration. Hydrophilic membrane ultrafilters with MW cutoffs of 3,000, 10,000 and 30,000 daltons were used. The control CNS medium was also divided into the same MW fractions as CMF.

Filtrates containing molecules of MW <3,000, 10,000 and 30,000 daltons were obtained by ultrafiltration. It was found that the filtrate containing molecules of MW <3,000 daltons did not significantly inhibit olfactory neurite outgrowth (Table 6). The filtrate containing molecules of MW <10,000 or 30,000 daltons, however, reduced the percentage of ORNs bearing neurites significantly. All filtrates did not significantly influence the attachment of the ORNs. The results suggest that the inhibitory substance(s) in CMF is of MW >3,000 daltons.

(2) Culture of ORNs on Cortical Astrocytes in Heat-treated CMF

Conditioned medium of fibroblasts and CNS medium were boiled at 100°C for 5 minutes prior to use in cultures.

Like CMF which had not been heat-treated, the boiled CMF resulted in a significantly lower percentage of neurite-bearing ORNs (50%) as compared to the control (62%) (Table 7). However, unlike CMF which had not been heat-treated, significantly less ORNs attached to the astrocytes. The morphology of the ORNs remained unchanged in the heat-treated CMF.

The experiment shows that the inhibitory effect of media

conditioned by fibroblasts on olfactory neurite extension is not significantly affected by heat treatment. The result suggests that the inhibitory substance(s) either is not a protein, or is a heat stable protein.

(3) Protein Assay

The total amount of protein in CMA, CMF and CNS medium was determined. It was found that the protein content of CMA (4.00 mg/ml) and CMF (4.48 mg/ml) were similar to that of CNS medium (4.32 mg/ml).

(4) Concentration-Activity Relationship

To examine whether the inhibitory effect of CMF was dose-dependent, the fraction of CMF containing molecules of MW >3,000 daltons was concentrated by filtering CMF through a membrane ultrafilter with a MW cutoff of 3,000 daltons. The retained fraction was initially concentrated 10 times, and then serially diluted and used.

It was found that the effect of CMF containing molecules of MW >3,000 daltons on ORN attachment to astrocytes was dose-dependent (Table 8) (Fig. 9B). There were significantly fewer cells attached to the substratum when CMF was concentrated from 1 to 2.5 times, and from 2.5 or 5 times to 10 times. However, the percentage of ORNs bearing neurites appeared not to be influenced by the concentrations of CMF (Table 8) (Fig. 9A). There was no significant reduction of the percentage as the concentration of CMF increased.

Table 2. Olfactory Neurite Extension on Various Substrata

Substrata	Percentage of ORNs Bearing Neurites	Neurite Length (μm)	Mean Number of ORNs Attached to the Substrata per c.s.
Cortical astrocytes	63 ± 4 (15)	188 ± 82 [80]	709 ± 124 (15)
OB astrocytes	65 ± 3 (6)	181 ± 71 [40]	634 ± 121 (6)
Fibroblasts	13 ± 4 (5)*	90 ± 48 [40]*	182 ± 40 (5)*

The ORNs were cultured on various substrata for 2 days, then labelled with RT97 and examined on a fluorescent microscope. The figures in parentheses and brackets represent the number of coverslips (c.s.) and the number of ORNs examined respectively. All results are presented as ± SD.

*: The percentage of ORNs bearing neurites, neurite length, and the mean number of ORNs attached to fibroblasts were all significantly different from that on cortical or OB astrocytes ($P<0.001$).

Table 3. Olfactory Neurite Extension on ONL glial Cells

Substrata	Percentage of ORNs Bearing Neurites	Neurite Length (μm)	Mean Number of ORNs Attached to the Substrata per c.s.
ONL glial cells	69 ± 2 (6)	187 ± 59 [40]	659 ± 132 (6)
Cortical astrocytes	68 ± 1 (4)	198 ± 58 [40]	764 ± 181 (4)

The ORNs were cultured on the substrata for 2 days, then labelled with RT97 and examined on a fluorescent microscope. Bovine pituitary extract was added at 100 μg/ml into the culture medium. The figures in parentheses and brackets represent the number of coverslips (c.s.) and the number of ORNs examined respectively. All results are presented as ± SD.

Table 4. ORN Culture in Conditioned Media

Substrata	Media	Percentage of ORNs Bearing Neurites	Mean Number of ORNs Attached to the Substrata per c.s.
Cortical astrocytes	CMF	55 ± 4 (8)[a]	650 ± 237 (4)
	CNS Medium	64 ± 3 (6)	743 ± 144 (6)
Fibroblasts	CMA	24 ± 5 (6)	296 ± 82 (6)[b]
	CNS Medium	29 ± 6 (5)	173 ± 51 (5)

The ORNs were cultured on astrocytes in CMF, or on fibroblasts in CMA for 2 days, then labelled with RT97 and examined on a fluorescent microscope. The figures in parentheses and brackets represent the number of coverslips (c.s.) and the number of ORNs examined respectively. All results are presented as ± SD.

a: The percentage of ORNs bearing neurites was significantly reduced in the presence of CMF. $P<0.001$ as determined by Student's t-test.

b: Significantly more cells attached to fibroblasts in the presence of CMA. $0.02>P>0.01$ as determined by Student's t-test.

Table 5. ORN Culture on cortical astrocytes with CMF->CNS Medium*

Media	Percentage of ORNs Bearing Neurites	Neurite Length (μm)	Mean Number of ORNs Attached to the Substratum per c.s.
CMF->CNS Medium	63 ± 2 (8)[a]	143 ± 53 [40][b]	948 ± 244 (8)
CNS Medium	68 ± 3 (8)	169 ± 53 [40]	792 ± 232 (8)

*: The astrocytes were treated with CMF for about 6 hours prior to the addition of ORNs. The ORNs were then grown for 2 days in CNS medium, labelled with RT97 and examined on a fluorescent microscope.

The figures in parentheses and brackets represent the number of coverslips (c.s.) and the number of ORNs examined. All results are presented as ± SD.

a: The percentage of cells bearing neurites was reduced on CMF-treated astrocytes. $0.01>P>0.001$ as determined by Student's t-test.

b: The ORNs cultured on CMF-treated astrocytes also extended shorter neurites. $0.05>P>0.02$ as determined by Student's t-test.

Table 6. ORN Culture on Cortical Astrocytes in Filtrates of CMF

MW Ranges of Filtrates	Filtrate	Percentage of ORNs Bearing Neurites	Mean Number or ORNs Attached to the Substratum per c.s.
<3 kD	CMF	63.4 ± 5.3 (6)	595 ± 193 (6)
	CNS Medium	69.0 ± 3.3 (6)	628 ± 128 (6)
<10 kD	CMF	62.6 ± 4.7 (5)[a]	706 ± 125 (5)
	CNS Medium	68.4 ± 2.1 (8)	676 ± 183 (8)
<30 kD	CMF	59.5 ± 5.1 (7)[b]	754 ± 278 (7)
	CNS Medium	69.0 ± 4.2 (6)	751 ± 254 (6)

Filtrates containing molecules of different MW ranges were obtained by filtering CMF and CNS media through membrane ultrafilters with MW cutoffs of 3 kD, 10 kD and 30 kD respectively. The ORNs were cultured on cortical astrocytes in the filtrate for 2 days, then labelled with RT97 and examined on a fluorescent microscope.

The figures in parentheses represent the number of coverslips (c.s.) examined. All results are presented as ± SD.

a: The percentage of ORNs bearing neurites was significantly reduced in CMF containing molecules of MW <10 kD. $0.02>P>0.01$ as determined by Student's t-test.

b: The percentage of ORNs bearing neurites was also significantly reduced in CMF containing molecules of MW <30 kD. $0.01>P>0.001$ as determined by Student's t-test.

Table 7. ORN Culture on Cortical Astrocytes in Heat-treated CMF

Heat-treated Media	Percentage of ORNs Bearing Neurites	Mean Number of ORNs Attached to the Substratum per c.s.
CMF	50 ± 8 (6)*	793 ± 118 (6)*
CNS Medium	62 ± 2 (6)	1051 ± 96 (6)

Conditioned medium of fibroblasts and CNS medium were boiled at 100°C for 5 minutes prior to use. Olfactory receptor neurons were then cultured on cortical astrocytes in heat-treated CMF or CNS medium for 2 days, labelled with RT97, and examined on a fluorescent microscope.

The figures in parentheses represent the number of coverslips (c.s.) examined. All results are presented as ± SD.

*: The percentage of ORNs bearing neurites and the mean number of ORNs attached were significantly reduced in the presence of heat-treated CMF. Both $0.01>P>0.001$ as determined by Student's t-test.

Table 8. Effect of Concentrated CMF on Olfactory Neurite Extension on Cortical Astrocytes

Number of Times CMF is Concentrated	Percentage of ORNs Bearing Neurites	Mean Number of ORNs Attached to the Substratum per c.s.
10	57 ± 5 (4)	152 ± 16 (4)[b]
5	55 ± 5 (4)	223 ± 50 (4)
2.5	62 ± 3 (4)	243 ± 50 (4)[a]
1	54 ± 5 (4)	358 ± 62 (4)

Conditioned medium of fibroblasts, concentrated 10 times initially by filtration through a membrane ultrafilter with a MW cutoff of 3,000 daltons, was serially diluted. The ORNs were cultured on cortical astrocytes in the concentrated CMF for 2 days, labelled with RT97 and examined on a fluorescent microscope.

The figures in parentheses represent the number of coverslips (c.s.) examined. All results are presented as ± SD.

a: The number of ORNs attached to astrocytes was significantly reduced when CMF was concentrated from 1 to 2.5 times ($0.05>P>0.02$).

b: Significantly fewer cells attached to astrocytes when CMF was concentrated from 2.5 or 5 times to 10 times ($0.02>P>0.01$ and $0.05>P>0.02$ respectively).

Fig. 3. SEM micrographs of cortical astrocytes. (A) Smooth surfaces of two astrocytes. Their boundary (arrowheads) is relatively regular. Bar=10 μm. (B) The upper half of the picture shows a surface of a cell which has many protrusions, while the lower half shows a smooth surface of another cell. The middle of the picture shows part of the boundary between the two cells (arrowheads). Their junction is formed by the interdigitation of their jagged edges at the contact site. Bar=1 μm. (C) There are numerous round or finger-like protrusions on the astrocytes. A cytoplasmic extension from an astrocyte (asterisk) with short and longer protrusions contacting adjacent cell(s). Bar=1 μm. (D) Longer protrusions from two astrocytes interdigitate with each other. The arrowheads indicate the outline of one of the astrocytes. Bar=10 μm.

Fig. 3

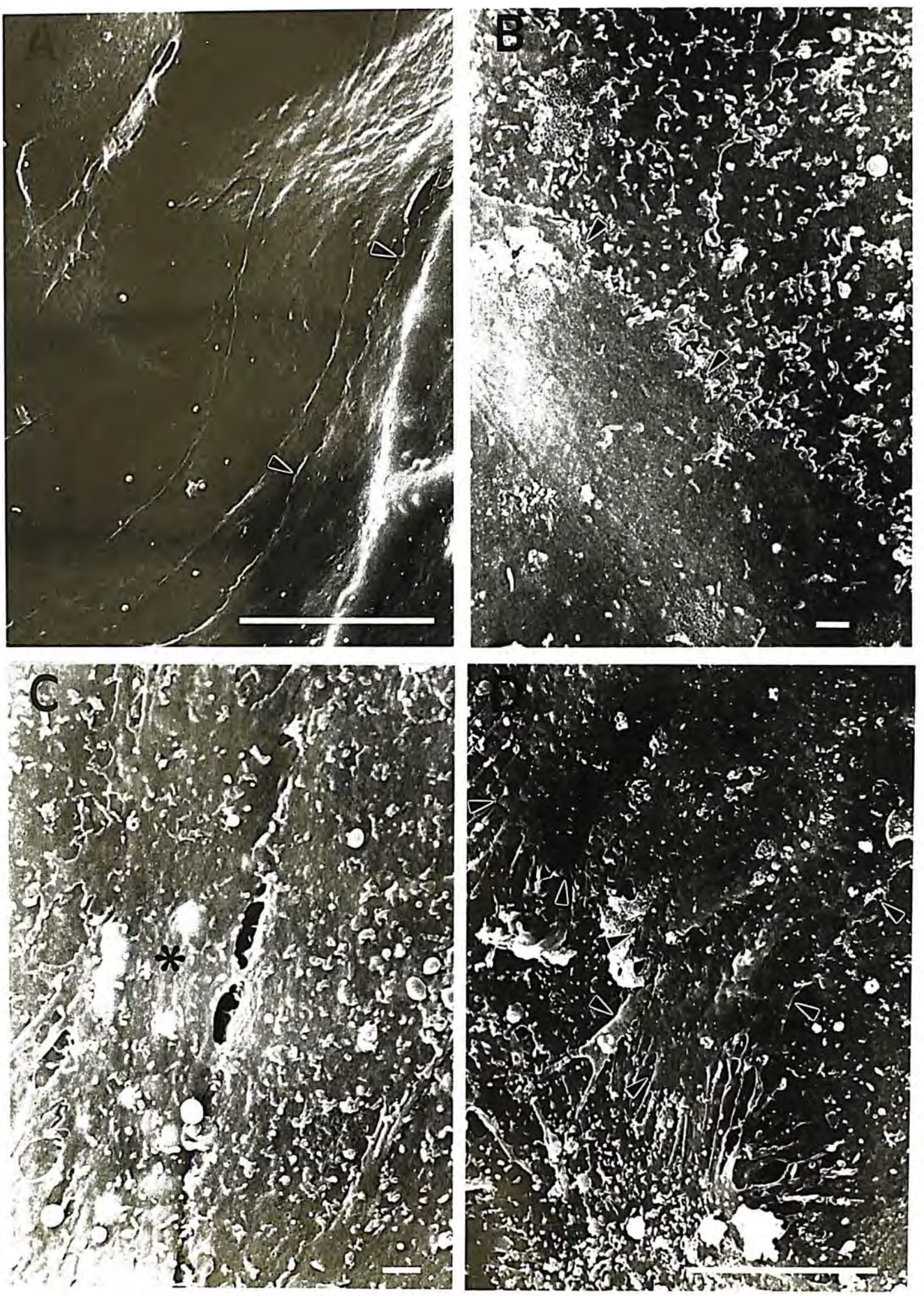


Fig. 4. TEM micrographs of cortical astrocytes. (A) Finger-like and round protrusions from an astrocytic surface. N: nucleus of the astrocyte. Bar=1 μm. (B) Smooth surface of an astrocyte. Astroglial filaments (arrowheads) are scattered in the cytoplasm. The arrows indicate the PLL coating of the petri dish. Bar=1 μm. (C) Two processes of astrocytes in contact. Note the fuzzy material at their contact sites (arrowheads). Astroglial filaments are present in bundles in the periphery of the lower cell (arrows). Bar=1 μm. (D) Four astrocytic processes (asterisks) in contact. Bar=1 μm.

Fig. 4

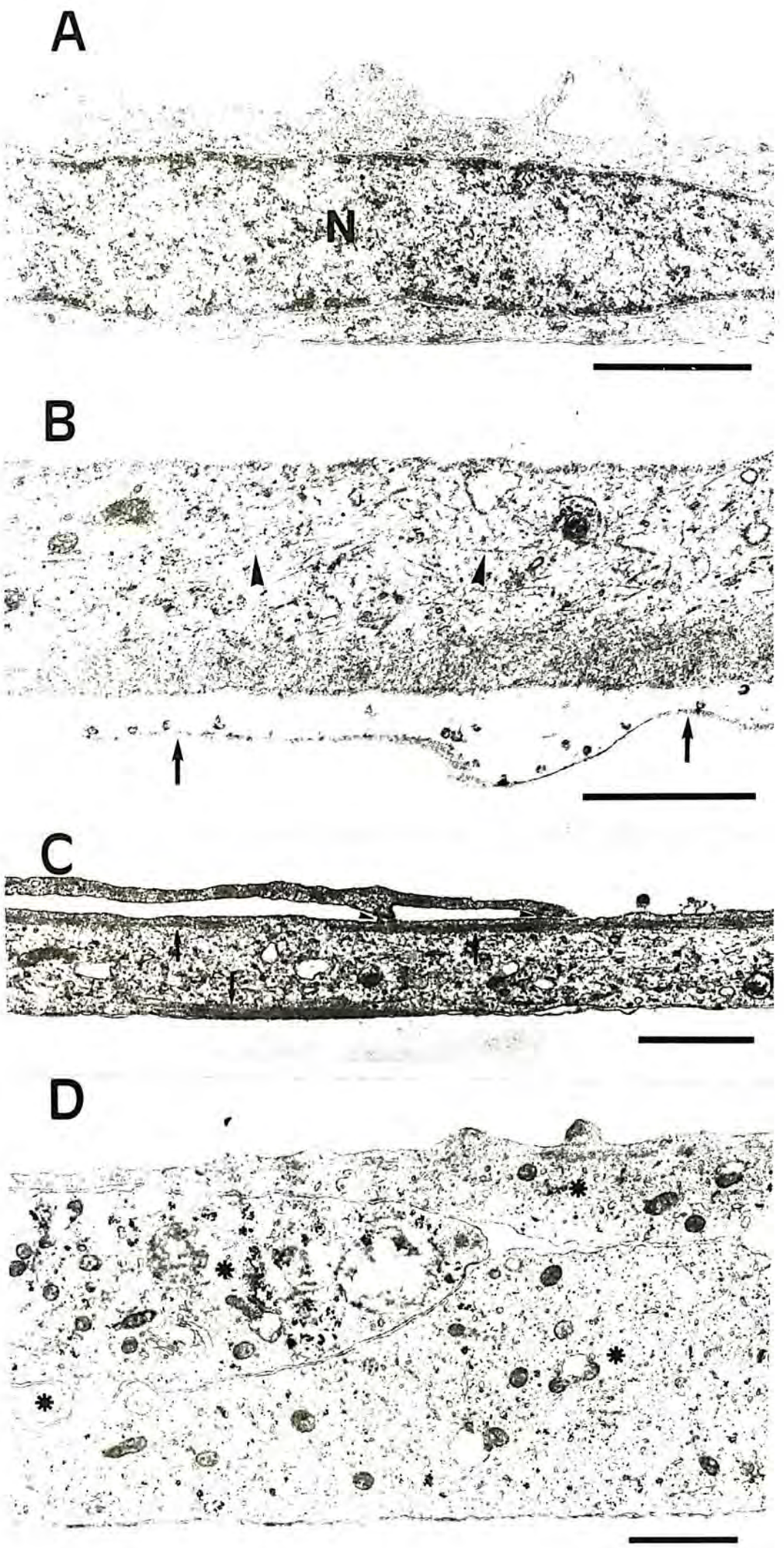


Fig. 5. SEM micrographs of ORNs cultured on cortical astrocytes. (A) An ORN with two processes. Note the rough surface of the cell body. Bar=10 μm. (B) A bipolar ORN whose dendrite ends in a swelling (arrowhead), and whose axon is long. The discontinuity of the processes with the cell body is an artifact. Bar=10 μm. (C) The short dendrite of this ORN ends in a flattened expansion which gives rise to filopodia. Bar=10 μm. (D) The arrowhead indicates a varicosity present on an axon extending from an ORN which has a smooth surfaced cell body. Bar=1 μm.

Fig. 5

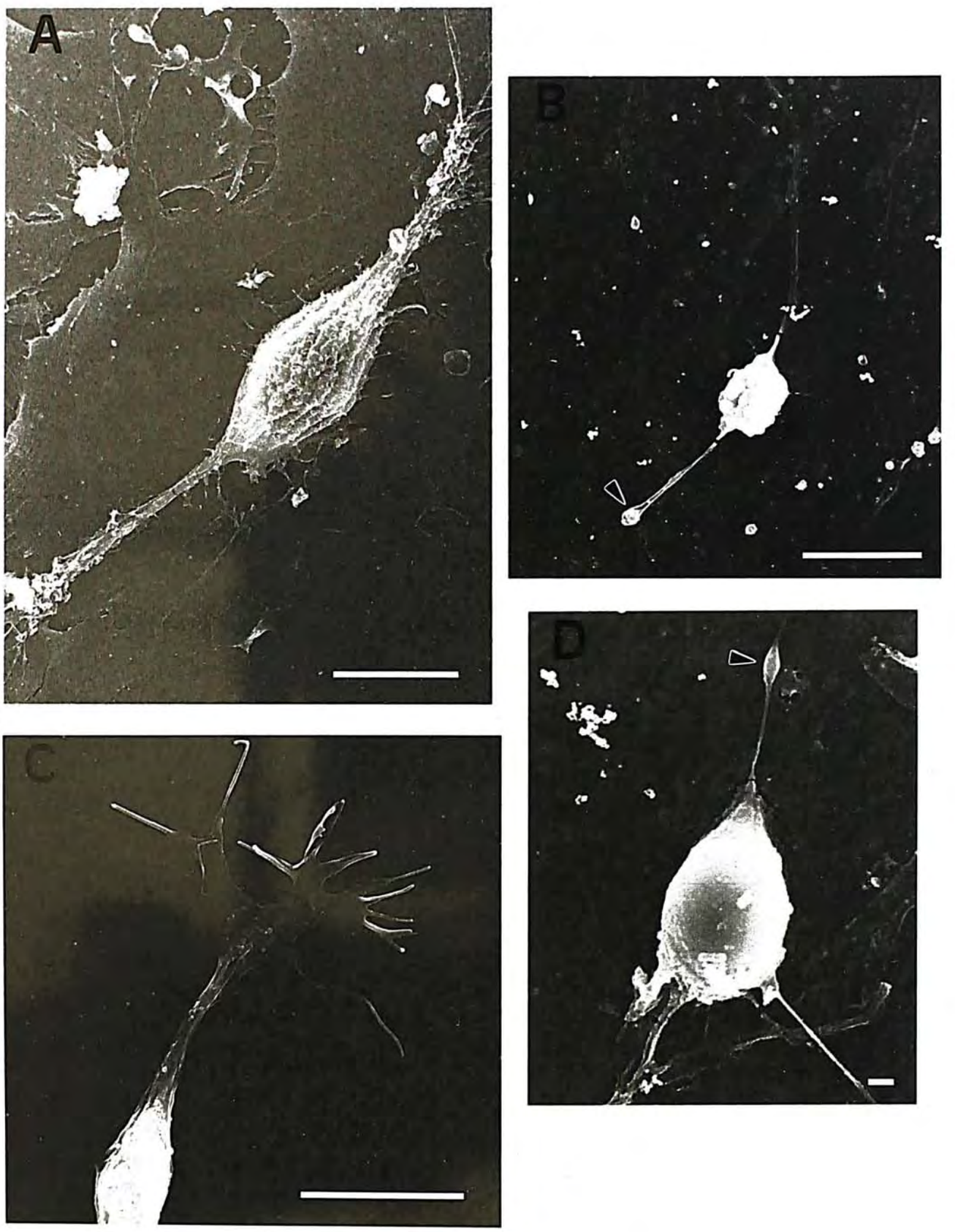


Fig. 6. TEM micrographs of ORNs cultured on cortical astrocytes. (A) An axon in contact with an astrocyte. Fuzzy material (arrowheads) is present at the axon-astrocyte contact site and on the astrocytic surface. Arrows: microtubules. Bar=0.1 μm. (B) An axon in apposition to a finger-like protrusion of an astrocyte. Arrows: microtubules. Bar=0.1 μm. (C) An ORN cell body in contact with an astrocyte. A coated vesicle (arrow) is present at their contact site. N: nucleus. Bar=1 μm. The inset shows higher magnification of the area enclosed by the rectangle. Bar=0.1 μm.

Fig. 6

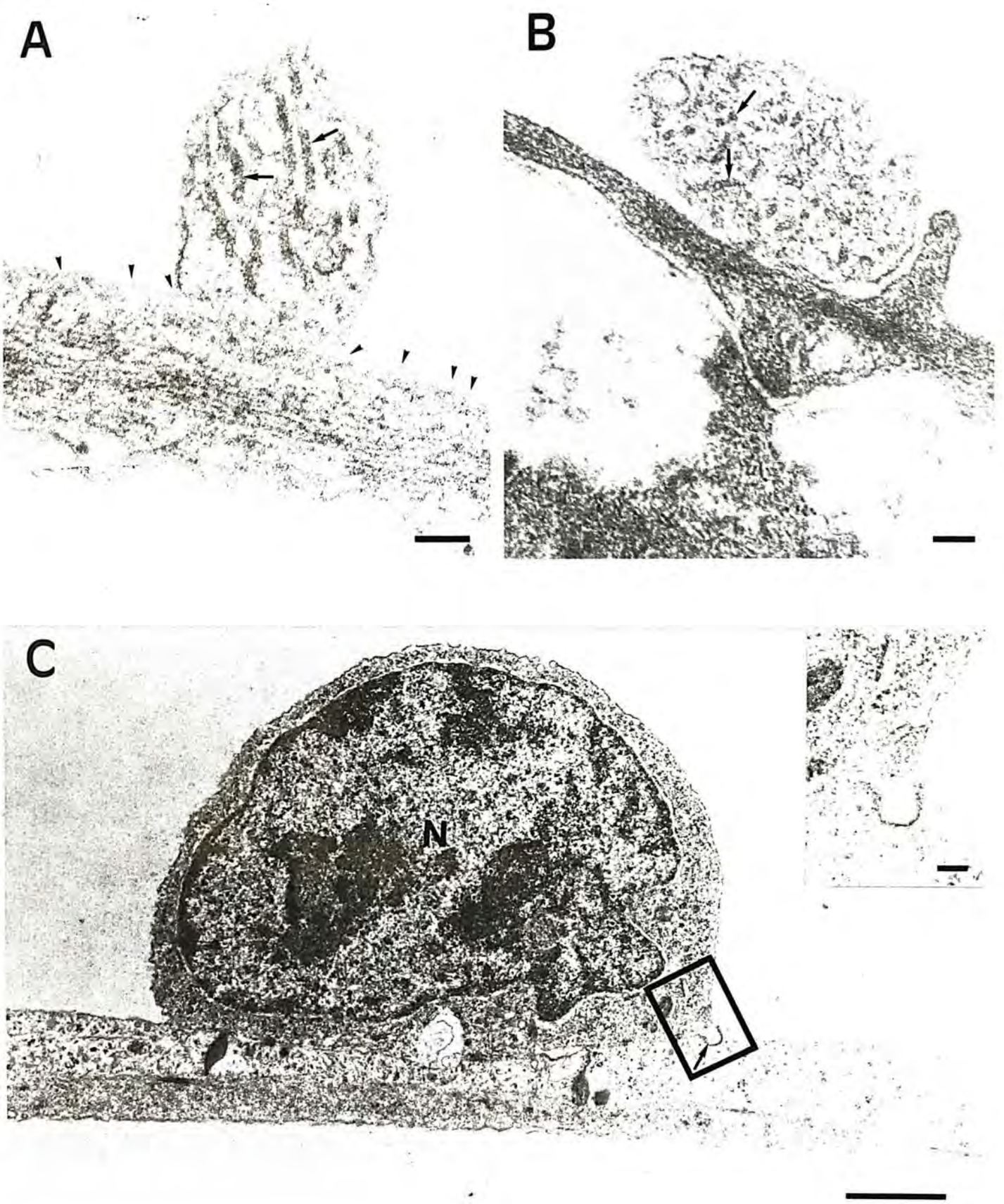
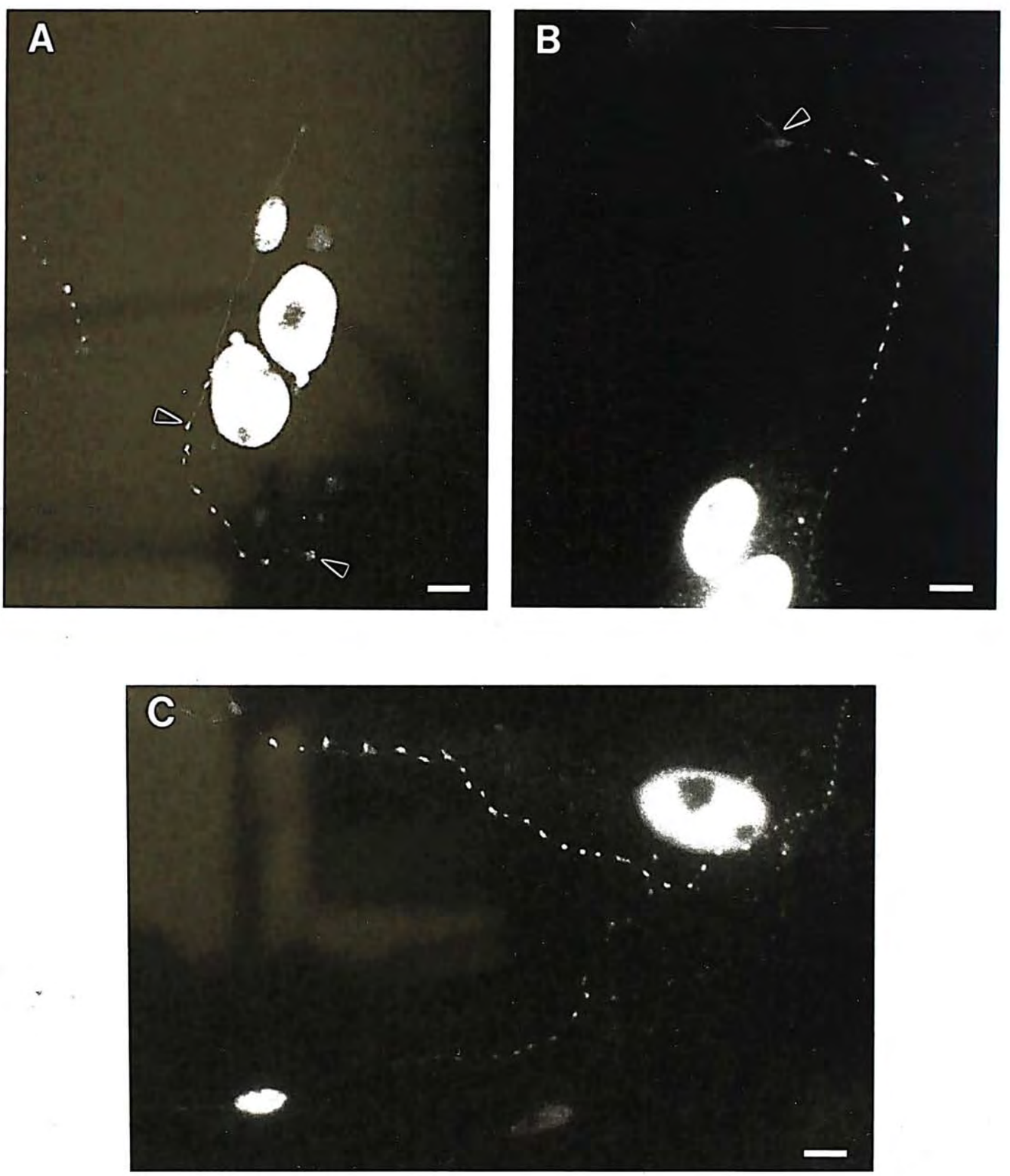


Fig. 7. Fluorescent micrographs of ORNs cultured on various cellular substrata. The ORNs are labelled with RT97. The nuclei of the cells are also stained as they contain histones which cross react with RT97. (A)-(C): ORNs on cortical astrocytes. (A) A bipolar ORN bearing a short dendrite and a long axon with varicosities (arrowheads). Bar=10 μm. (B) An axon ending in a flat dilatation (arrowhead). Bar=10 μm. (C) An ORN with a long and bifurcated axon often seen on cortical astrocytes. Bar=10 μm. (D) On OB astrocytes: an ORN with a long axon which has many varicosities and bifurcations. Bar=10 μm. (E) On fibroblasts: an ORN with a short and smooth looking axon. Bar=10 μm. (F) On ONL glial cells: An ORN cell body (arrowhead) giving rise to a long axon with many varicosities and bifurcations. Bar=10 μm.

Fig. 7



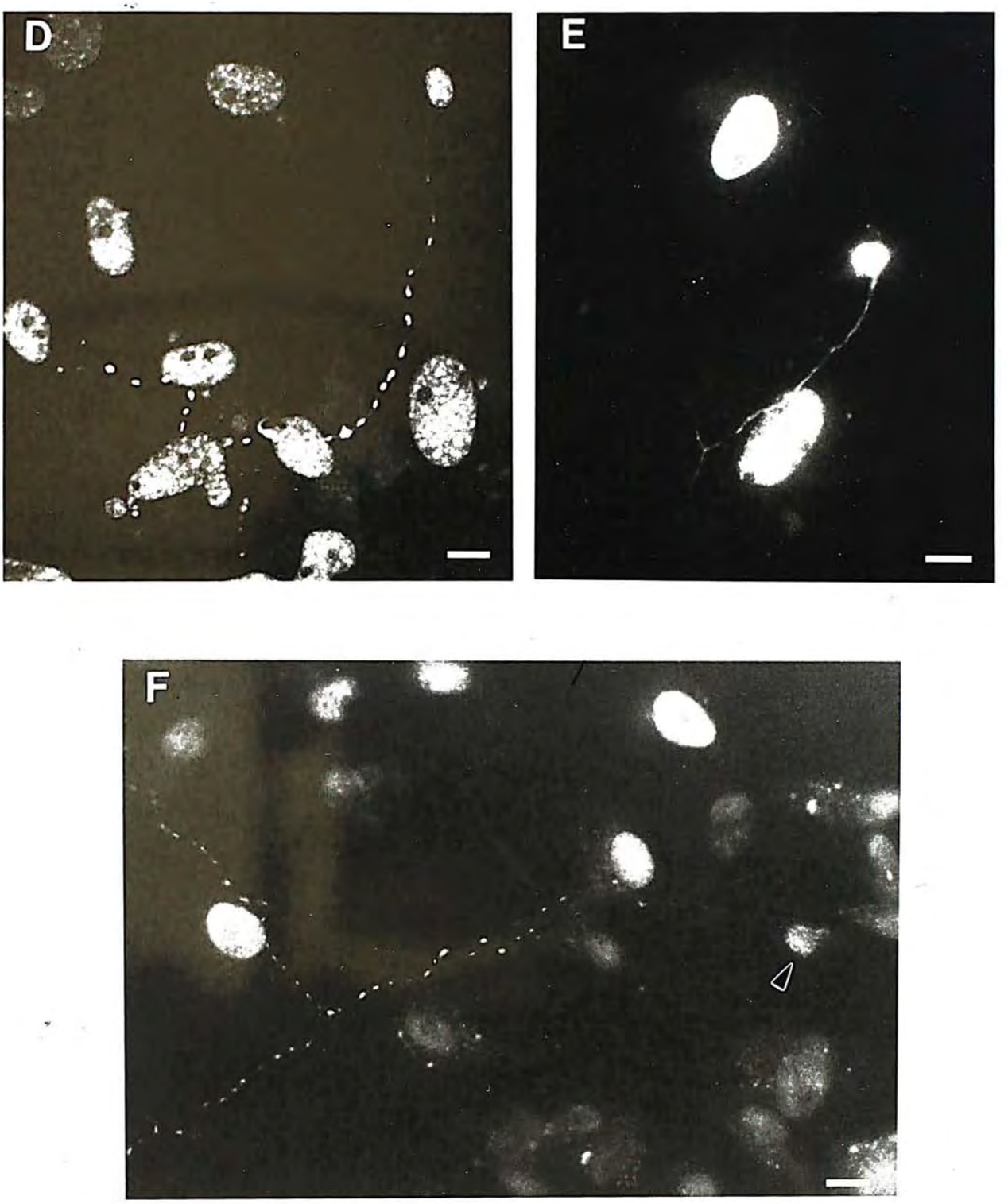
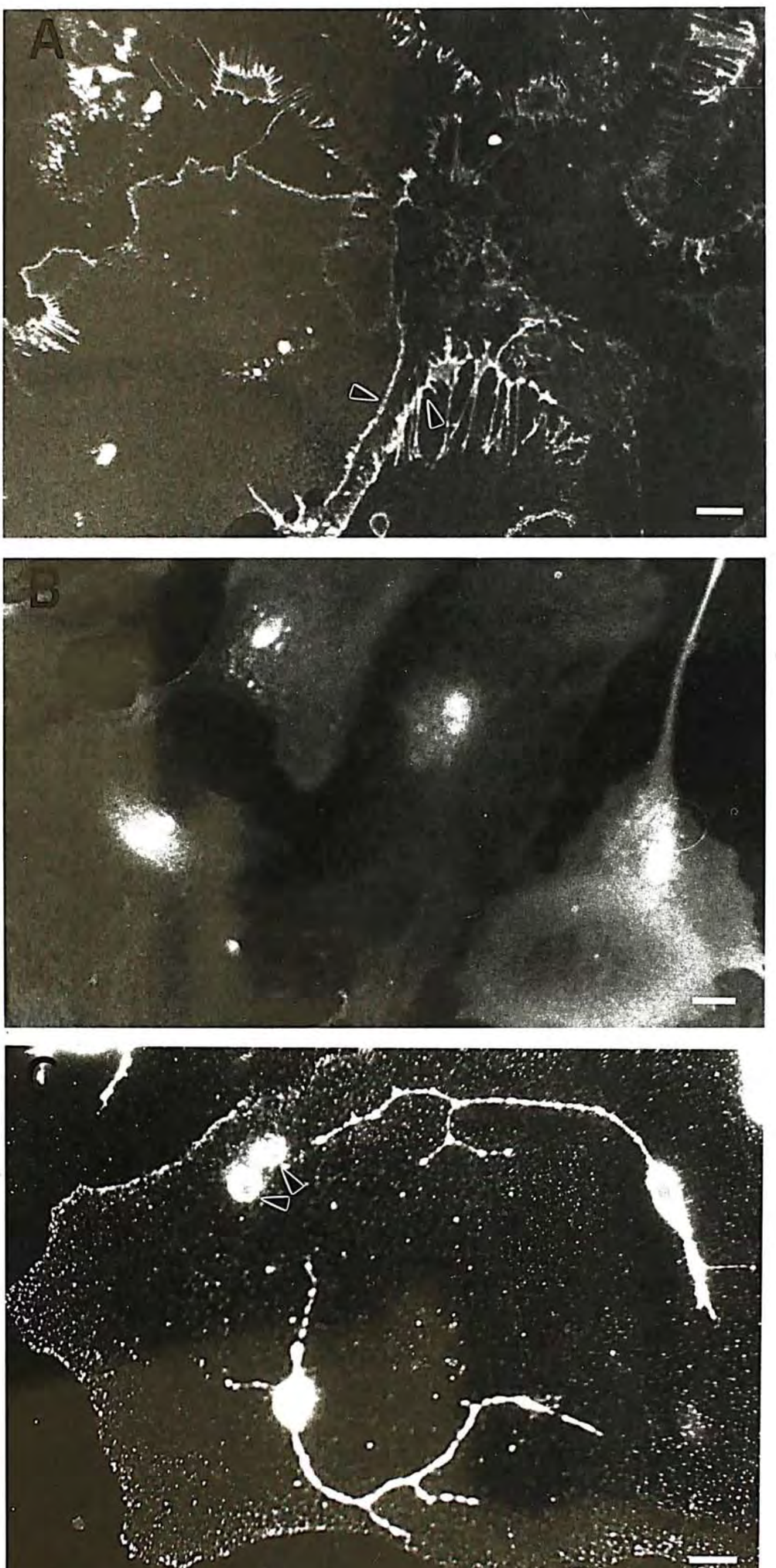

D
E
F

Fig. 8. Fluorescent micrographs of cortical astrocytes, ORNs and fibroblasts stained for CAMs. (A) Cortical astrocytes stained for N-CAM. The molecule is present in a punctate form all over the astrocytic surface. The staining is more intense on the edges (arrowheads). Bar=20 μm. (B) Cortical astrocytes stained for N-cadherin. The molecule is also present all over the astrocytic surface. Most nuclei are heavily stained as shown in the picture, while some are weakly labelled (not shown). Bar=20 μm. (C) N-CAM staining of part of a cortical astrocyte with ORNs growing on it. The two bipolar ORNs are intensely stained. There are also two ORN cell bodies bearing no neurites (arrowheads). Bar=10 μm. (D) Fibroblasts stained for N-cadherin. The molecule is present throughout the cell surfaces. Some nuclei are heavily stained (arrowhead). Bar=20 μm. (E) Fibroblasts stained for N-cadherin. Some nuclei are barely labelled (arrowhead). Bar=20 μm.

Fig. 8



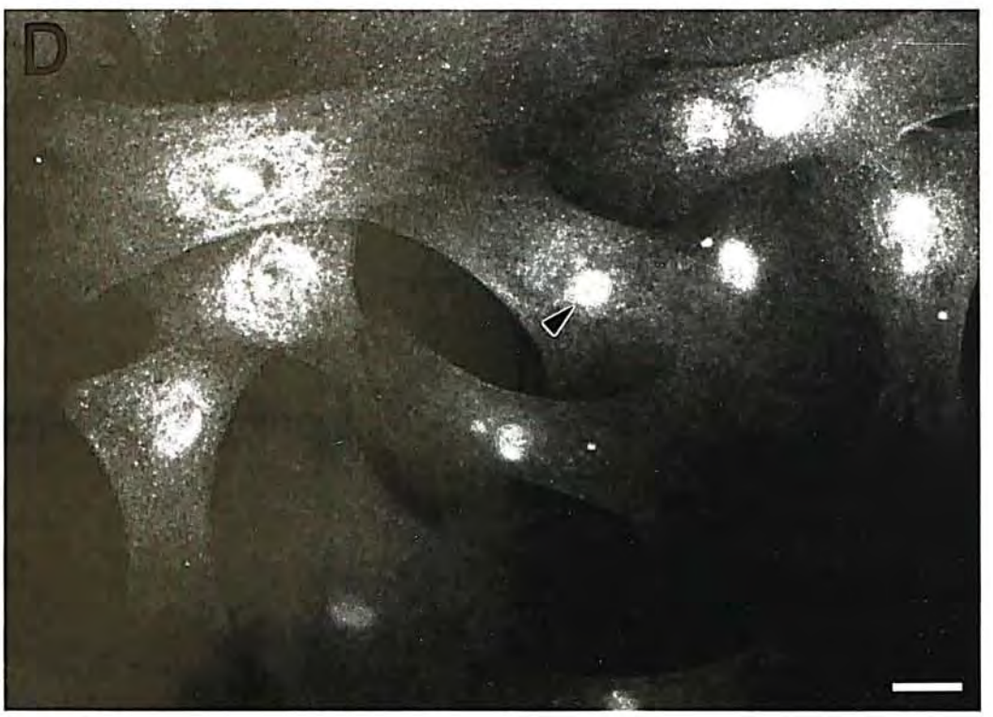

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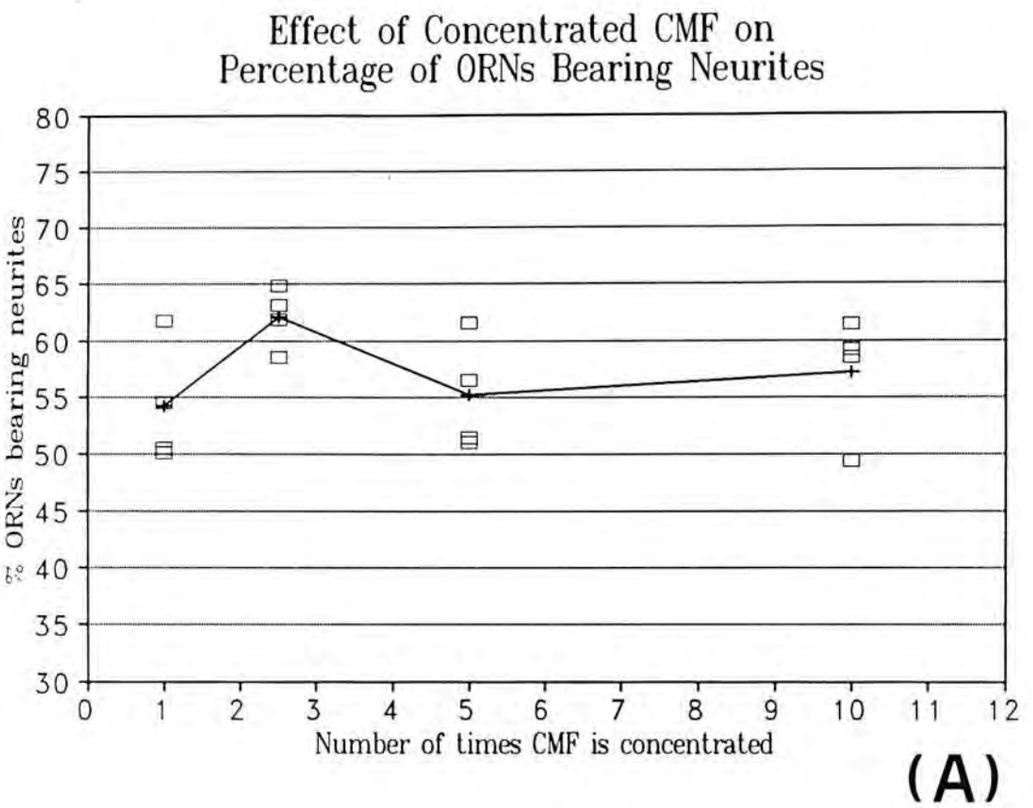
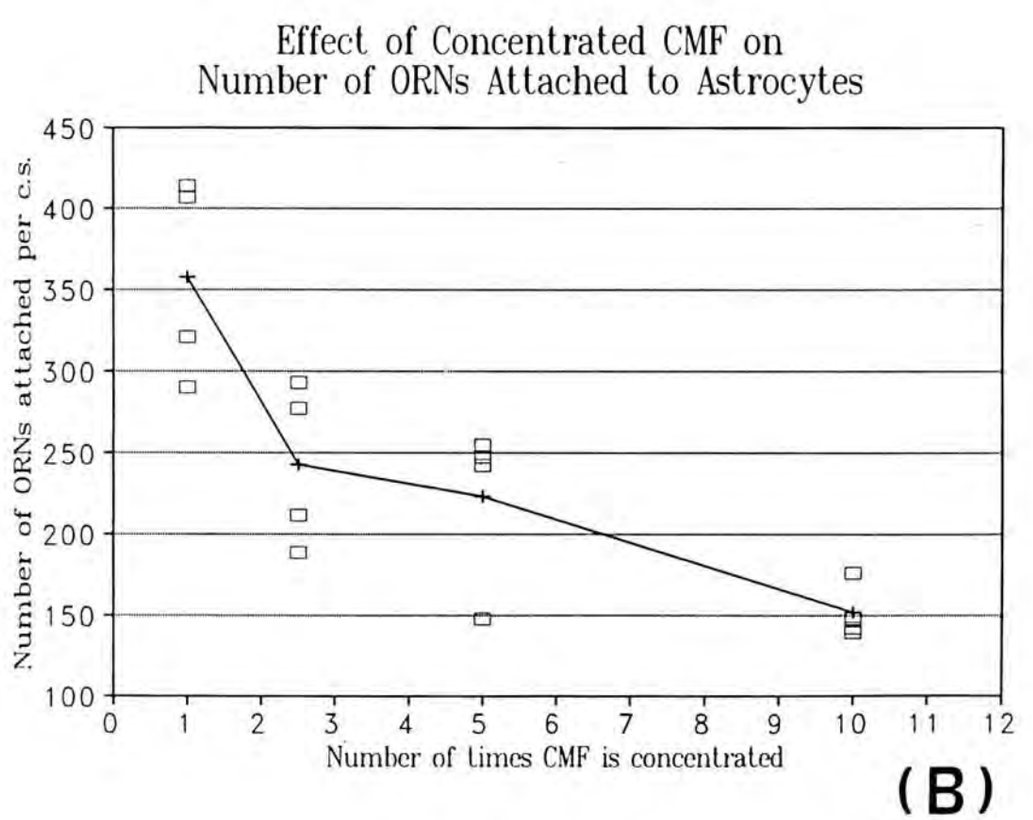
Fig. 9. Concentration curves. Four coverslips were examined at each concentration. (A) Effect of concentrated CMF on the percentage of ORNs bearing neurites. (B) Effect of concentrated CMF on the mean number of ORNs attached to astrocytes.

Fig.9


Effect of Concentrated CMF on
Percentage of ORNs Bearing Neurites
% ORNs bearing neurites
80
75
70
65
60
55
50
45
40
35
30
0 1 2 3 4 5 6 7 8 9 10 11 12
Number of times CMF is concentrated

(A)


Effect of Concentrated CMF on
Number of ORNs Attached to Astrocytes
Number of ORNs attached per c.s.
450
400
350
300
250
200
150
100
0 1 2 3 4 5 6 7 8 9 10 11 12
Number of times CMF is concentrated

(B)

DISCUSSION

The purpose of this study is to investigate some aspects of neuronal differentiation using a cell culture model of olfactory receptor neurons. The results of the study confirm and extend the finding that ORNs can differentiate morphologically on cultured cortical astrocytes. Furthermore, olfactory differentiation, denoted by neurite outgrowth, also occurs when ORNs are cultured on OB astrocytes and ONL glial cells. Fibroblasts, however, are much less effective in promoting olfactory neurite extension. Culture medium conditioned by fibroblasts inhibits olfactory neurite extension on astrocytes. This inhibition is mediated by a substance(s) of MW greater than 3,000 daltons, which acts by adsorbing to the substratum.

Scanning and transmission electron microscopy conducted in this study showed that ORNs can extend axons and dendrites on cortical astrocytes. Like mature ORNs <u>in vivo</u>, cultured bipolar ORNs had pear-shaped cell bodies of about 10 μm in diameter, with the longer of the two processes being the axon. The axons had a diameter less than 0.5 μm, and had varicosities along its length. Transmission electron microscopy showed that microtubules were present within the axons. On SEM the dendrites were shorter and thicker. Taking into account the additional data from the present study, and information from previous reports (Noble et al., 1984a; Chuah et al., 1991), it can be concluded that ORNs can differentiate morphologically on cortical astrocytes.

Although recent studies (Moran et al., 1982; Rowley et al.,1989) have discovered a second type of neuron, the microvillar cell, in the OE, this type of cell was unable to be identified under light

microscopy. This is because RT97, an antibody against neurofilaments, was used to stained for the neurons. Apical cell modifications, such as microvilli and cilia, would not be stained by this antibody, and hence these distinguishing features could not be observed. In the case of electron microscopy, a large number of cells were lost during processing. Considering that microvillar cells are present in a ratio of 1:20 with receptor neurons (Rowley et al., 1989), very likely few if any microvillar cells remained on the coverslips by the time the specimens were ready for observation.

The observation that the surface of cultured astrocytes can be either smooth or uneven has deen reported by Grierson and his collegues (1990). They showed by phase contrast microscopy and immunofluorescence that hypothalamic neurons preferred the flat areas, and few neurons were present on the rocky ones. In the present study, there also appeared to be more axons observed on the smooth astrocytic surface, although no quantitative study was done to confirm this.

Transmission electron microscopy in this study showed that fuzzy material was present on discrete sites of astrocytic surface. This material appeared similar to that observed by Ard and Bunge (1988) in their study on cultured cortical astrocytes. They found by transmission electron microscopy that fibrillary material was present on some regions of astrocytes, and these stained positively for LN by immunofluorescence. Similar to their findings, the fuzzy material in the present study was not related with any cytoskeletal filaments, thus ruling out the possibility that this deposit could be a type of CAM.

In this study, the morphology and neurite outgrowth from ORNs

cultured on cortical astrocytes, OB astrocytes, ONL glial cells and fibroblasts have been compared. The three sources of glial cells resulted in morphologically similar ORNs. In contrast, ORNs cultured on fibroblasts produced shorter neurites which had fewer bifurcations and varicosities. This morphology is probably regulated by surface interaction between ORNs and the substratum rather than soluble factors since substitution of the culture medium with CMA failed to produce a change in cell form. Results from previous studies (Noble et al., 1984b) agree with the present findings. It was found that both cerebellar and spinal cord neurons assumed different morphologies when they were cultured on astrocytes or fibroblasts. Exposure of neurons cultured on astrocytes to medium conditioned by fibroblasts and vice versa did not change the morphology.

Quantitative results in this study showed that cortical astrocytes, OB astrocytes, and ONL glial cells were similarly effective in supporting neurite growth. This appears to contradict findings from a study by Denis-Donini et al (1984) in which they found that mesencephalic neurons grown on either striatal or mesencephalic astrocytes developed different morphologies. They attributed the difference to the fact that striatal and mesencephalic astrocytes possess different antigenic properties. Evidence from other parts of the nervous system such as the optic nerve lends further support to the notion that there is considerable heterogeneity among astrocytes (Raff et al., 1983; Miller and Raff, 1984).

In the present study, it was not surprising to find that OB astrocytes elicited a similar rate of neurite extension as the cortical

astrocytes, since OB astrocytes are known to be derived from the CNS (Doucette, 1984, 1989). What was perhaps surprising was that the ONL glia did not promote more neurite growth than the other two groups mentioned above. One explanation is that the ONL glial cells are functionally similar to cortical and OB astrocytes. This seems unlikely since ultrastructural evidence indicates that the ONL contains a group of glial cells, better known as ensheathing cells, which are peripherally derived and are morphologically different from cortical and OB astrocytes (Doucette, 1984, 1989). Glial heterogeneity was indeed demonstrated in the preparation of ONL glia in vitro. Because the ensheathing cells formed 73% of the ONL glial cell cultures, one cannot rule out the possibility that the increased stimulatory effect of the ensheathing cells may have been masked. An obvious solution would be a refinement of the technique such that one can yield 100% pure ensheathing cell cultures. With the technique that was used in this study, it would seem that a prolonged culture time could result in a completely confluent layer. This alternative may be problematic since prolonged culture could alter antigenic properties of the cells as was found with astrocytes (Smith and Miller, 1991). The difficulty in obtaining a completely confluent layer of ensheathing cells in this study may be due to the absence of axonal elements. Previous researchers found that cell proliferation in the OB during development was greatly enhanced by ingrowing olfactory axons (Piatt, 1951; Stout and Graziadei, 1980).

Unlike the astrocytes, the percentage of ORNs that extended neurites on fibroblasts was decreased. Immunofluorescent staining

showed that N-CAM and N-cadherin were present on cortical astrocytes, while fibroblasts had only N-cadherin. Thus the reduced neurite growth on fibroblasts can be partly attributed to the absence of N-CAM.

Since neurite outgrowth is known to be regulated not only by cell-substratum interaction, but also soluble factors, experiments utilizing medium conditioned by fibroblasts or astrocytes were conducted. The evidence points to the existence of an inhibitory substance(s) rather than the lack of neurite promoting factors in CMF. This is indicated by the fact that the percentage of ORNs extending neurites on astrocytes was decreased in the presence of CMF. Furthermore, pretreatment of astrocytes with CMF also resulted in a reduction of neurite outgrowth, suggesting that the inhibitory substance(s) acted by adsorbing to the astrocytes. It has been shown previously that some soluble substances affect neuronal survival and neurite growth by adsorbing to culture substrata. For example, Lander et al (1982) showed that rat sympathetic neurons extended neurites on polylysine-coated dishes treated with medium conditioned by corneal endothelial cells, but not on untreated dishes.

At this stage it is uncertain to which part of the astrocytes does the inhibitory substance(s) adsorb. It may be acting independently of the CAMs, or it may be binding to the CAMs in such a way as to neutralize the neurite outgrowth-promoting properties of the molecules. The latter suggestion may be likely in view of increasing evidence that soluble factors are able to modify the level of specific membrane glycoproteins. For example, NGF has been shown to increase the expression of N-CAM and L1 in PC12 cells and cultured Schwann cells

respectively (Prentice et al., 1987; Seilheimer and Schachner, 1987).

Analysis of the CMF showed that the inhibitory substance(s) has a MW greater than 3,000 daltons. It is heat stable, suggesting that it is either not a protein(s), or is a heat stable protein(s). The protein content of CMA, CMF and CNS medium ranged from 4.00-4.48 mg/ml; but these values cannot show conclusively whether a protein substance(s) had been added during the culture period. The protein assay is not sensitive enough to detect minute differences in protein content resulting in the presence of the inhibitory substance(s).

The effect of CMF on the percentage of ORNs extending neurites was not dose-dependent as shown by the range of concentrations used in this study (Fig. 9A). Perhaps in CMF the amount of inhibitory substance(s) was already high enough such that the substance(s) had saturated all the binding sites on the astrocytes, so that neurite inhibition was not increased upon exposure to the concentrated CMF.

Although the scope of this work is focused on neuronal differentiation, the number of ORNs on each coverslip in the various experiments has also been counted. The number of ORNs per coverslip has been regarded as an adhesion assay of ORNs on the substrata. However, it should be noted that other studies have adopted a more rigorous assay in determining cell attachment to substratum which involves shaking the cultures at 350 rotations per minute prior to counting the number of attached cells (Rousselet et al., 1990). Such a procedure was not adopted in this study because ORNs appeared to attach less firmly to substrata than other neurons. It has been shown that attachment of ORNs to the substratum is not mediated by common

attachment factors such as PLL, LN or FN (Chuah et al., 1991) which have proved to be extremely effective for dorsal root ganglia, sympathetic ganglia, spinal cord and retina neurons (Rogers et al., 1983).

From the results it is uncertain whether the phenomena of cell attachment and neurite extension are mediated by the same molecule(s). Neither can one assume that the same molecule(s) is involved in ORN attachment to astrocytes and fibroblasts. What is more certain is that the attachment factor is likely to be a protein(s) as demonstrated by the decreased number of cells on astrocytes following heat treatment of CMF. It is clear that further investigations are needed to elucidate the nature of the molecule(s) mediating ORN attachment to substratum.

In summary, this work illustrates the complex cellular interaction that regulates neuronal differentiation. Cells can influence each other via interaction of integral membrane proteins, secretion of soluble factors, or a combination of these two mechanisms. How this intricate interaction triggers internal cellular processes to result in morphological differentiation will be another exciting area of research.

CONCLUSIONS

Olfactory receptor neurons are able to differentiate morphologically on cultured cortical astrocytes. They can also extend neurites on OB astrocytes and ONL glial cells. However, fibroblasts are less effective in mediating olfactory neurite outgrowth. Culture medium conditioned by fibroblasts inhibits olfactory neurite extension on cortical astrocytes. The inhibitory substance(s) in CMF has a MW greater than 3,000 daltons, and acts by adsorbing to the substratum.

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